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Original Article

Investigating the Role of HSF1 in the Folding of Oncogenic ALK Fusion Proteins

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Abstract. The chimeric gene fusions EML4-ALK and KIF5B-ALK have been previously identified as potent oncogenic drivers in non-small-cell lung carcinoma (NSCLC), with malignant transformation arising from the constitutive tyrosine kinase activity of their ALK domains. Despite the initial success of ALK-positive lung cancer therapies involving ALK and Hsp90 inhibitors, the susceptibility of these approaches to acquired drug resistance and cytotoxicity call for alternative strategies to attenuate ALK-driven oncogenesis. This study investigates the potential of targeting the chaperone-mediated folding mechanisms of these ALK fusions via the key heat shock response transcription factor HSF1, in light of reports that these oncoproteins were intrinsically unstable and prone to misfolding. It was found that expression levels of the isoform EML4-ALK variant 1, as well the novel fusion KIF5B-ALK, are particularly susceptible to Hsp90 inhibition, proteasome degradation and protease digestion, indicating high structural instability and dependence on chaperone activity. Overexpression of KIF5B-ALK was shown to induce HSF1 trimerization and activation, while both EML4-ALK and KIF5B-ALK were shown to increase HSF1 transcriptional activity independent of their kinase function. However, overexpression of HSF1 did not lead to any increase in ALK protein levels. This suggests that inhibiting HSF1 activity may have to be combined with other ALK-directed strategies in order to have any significant therapeutic effect in NSCLCs.

Keywords: lung cancer, fusion oncogene, anaplastic lymphoma kinase, echinoderm microtubule-associated protein like-4 (EML4), kinesin family member 5B (KIF5B), heat shock factor 1

Introduction

Gene fusion due to chromosomal translocations counts among the important mechanisms for carcinogenesis. One such common fusion involves anaplastic lymphoma kinase (ALK), a gene encoding for a receptor tyrosine kinase normally involved in neuronal growth and development. ALK was first discovered in patients with anaplastic large cell lymphoma (ALCL), as part of an oncogenic chimeric protein incorporating the N-terminal domain of nucleophosmin (NPM) and the C-terminal intracellular kinase domain of ALK (Gerber & Minna, 2010). Since then, ALK has been found to be involved in numerous other chromosomal rearrangements that drive tumorigenesis in non-Hodgkin's lymphomas, neuroblastomas, inflammatory myofibroblastic tumors and non-small cell lung cancer (NSCLC) (Li et al., 2013).

Of particular interest in this study is the role of ALK fusion oncoproteins prevalent in NSCLCs, which are estimated to comprise approximately 80-85% of all lung cancer cases. They constitute a particularly lethal form of carcinoma, with low sensitivity to cytotoxic drugs, a median patient survival time of less than one year and an average survival rate of less than 15% within five years. Interestingly, ALK fusion rearrangements have been found to be a potent oncogenic driver in a subset of NSCLCs (13%), both *in vivo* and *in vitro* (Shaw et al., 2009).

ALK fusion oncoproteins are constitutively active due to ligand-independent homodimerization of the ALK tyrosine kinase domain, which is mediated by the self-association of the N-terminal fusion gene partner. Downstream consequences of constitutive ALK activation are high activation states of the Raf/MEK/ERK and Jak/STAT signaling pathways and, eventually, malignant transformation (Ardini et al., 2010). The most common 5'-fusion partner in ALK-positive NSCLCs is the echinoderm microtubule-associated protein like-4 (EML4), a gene belonging to a class of microtubule destabilizers whose precise function remains unknown. This chimeric aberration results from a small inversion on the short arm of chromosome 2p. This fusion invariably includes exons 20-29 of ALK (which encode for the entire intracellular kinase domain) with varying proportions of the EML4 gene at the N-terminus. Tumors harboring EML4-ALK (about 6% of NSCLCs) are associated with distinct histological features (e.g. acinar histology) and tend to be highly sensitive to ALK-specific tyrosine kinase inhibitors (Soda et al., 2007). In addition to EML4, another novel fusion partner has also been recently identified in a small subset of NSCLC patients via immunohistochemical diagnostic screens: kinesin family member 5B (KIF5B), which usually codes for part of a motor protein complex associated with microtubular transport (Takeuchi et al., 2009; Wong et al, 2011). To date, the properties of KIF5B-ALK-positive NSCLCs and potential therapeutic strategies remain poorly understood.

Given the crucial role of the tyrosine kinase domain in oncogene addiction in these fusion-type NSCLCs, ALK has been an obvious therapeutic target. In preclinical models, ALK inhibitor drugs such as crizotinib have been reported to greatly attenuate growth and induce apoptosis in EML4-ALK-positive lung cancer cells, and crizotinib has been approved for clinical use in NSCLC (Takezawa et al., 2011). However, the effectiveness of ALK inhibitor therapy is ultimately limited by acquired drug resistance, which arises due to secondary and gatekeeper mutations in the inhibitor-binding domains of the target kinase, or activation of alternative signaling pathways that bypass dependence on ALK per se (Katayama et al., 2011).

Another potential therapeutic strategy is to target the chaperone-mediated folding via heat shock protein 90 (Hsp90) inhibitors. ALK fusion proteins are somatically acquired upon chromosomal translocation and do not exist naturally. Furthermore, the domains derived from the cytoskeletal EML4 and KIF5B proteins havea relatively open structure with hydrophobic residues exposed. This likely increases the dependence of these fusion oncoproteins on the activity of Hsp90 and other chaperones for proper folding and stabilization. Indeed, EML4-ALK has been shown to be associated with Hsp90 in cancer cells and to be rapidly degraded upon Hsp90 inhibition via the proteasome. Hence, tumor regression in EML4-ALK-positive cancers has been reported in response to treatment with Hsp90 inhibitors such as borzetomib or IPI-504 (Normant et al., 2011). Nonetheless, as chaperones are ubiquitous and key regulators of folding, activity and sorting of protein substates, Hsp90 inhibition runs the risk of high cytotoxicity. The limitations of these existing therapies thus raise the need for alternative strategies that might similarly exploit the chaperone-dependent folding of ALK fusion oncokinases while bypassing issues of drug resistance and detrimental off-target effects.

Apart from Hsp90 itself, an emerging folding pathway target for cancer therapy is heat shock factor 1 (HSF1) – the transcription factor which serves as the master regulator of the cytoprotective heat shock gene response. HSF1 regulates a genome-wide transcriptional program that, in addition to restoring the normal protein folding environment in the event of proteotoxic stress, also coordinates signaling pathways and modulates metabolism to ensure prolonged cellular survival (Hayashida et al., 2006). HSF1 has been shown to be highly activated in response to various stressors other than hyperthermia, including ischemia and hypoxia (conditions which are also prevalent in tumors) (Dai et al., 2007). Upon cellular stress, HSF1 homotrimerizes and is hyperphosphorylated in key residues before translocating into the nucleus and inducing transcription of the Hsps. HSF1 has been implicated in malignant transformation by coordinating a transcriptional network of key cellular functions such as glucose metabolism, proliferation, survival and protein synthesis (Whitesell & Lindquist, 2009). In light of this, inhibiting HSF1 activity emerges as an alternative therapeutic strategy for disrupting the chaperone-mediated stabilization of the EML4-ALK variants and KIF5B-ALK, thereby inducing their misfolding and shifting the balance of oncoprotein turnover towards degradation. Hence, the aim of the current study is to determine and compare the intrinsic instability of different ALK fusion proteins and assess the viability of HSF1 as a possible target for inhibition and attenuation of ALK fusion protein expression.

Materials and Methods

Plasmid constructs and mutagenesis

pcDNA 3.1-EML4-ALK v3a, pcDNA 3.1-EML4-ALK v3b, and pcDNA3.1-KIF5B-ALK were kindly provided by Dr. Maria Wong (The University of Hong Kong). pcDNA 3.1-FLAG-HSF1 was provided by Dr. Stuart Calderwood (Harvard Medical School) (Addgene Plasmid #32537). The pGL3 (Promega) luciferase reporter vectors Hsp70B/Luc (containing the Hsp70B promoter sequence upstream of the firefly luciferase gene) and 3xHSE/Luc (containing three copies of the idealized 15-base-pair HSE sequence AGAACGTTCTAGAAC linked tandemly in alternating orientation) were provided by Dr. Wulf Paschen (Duke University Medical Centre).

The expression plasmid containing the larger, more unstable variant EML4-ALK v1 (pcDNA3.1-EML4-ALK v1) was generated by inserting an 819-bp sequence (synthesized by ShineGene) between nucleotide positions 667 and 668 of EML4-ALK v3b. Briefly, the fragment upstream of this internal sequence was cloned from the pcDNA3.1-EML4-ALKv3b template using the primers 5'-AGCAGAGCTCTCTGG-3' (named pcDNA3/F) and 5'-AATATATTCTCCTTCTTGGTTGATGATG-3'. Similarly, the downstream fragment was cloned using the primers 5'-TGGGAAAGGACCTAAAGTGTACCGCCGG-3' and 5'-AGGCACAGTCGAGGC-3' (named pcDNA3/R). The internal sequence (with a 5' end complementary to the 3' end of the upstream fragment) was fused to the upstream fragment by fusion PCR with the primers pcDNA3/F and 5'-CCGGCGGTACACTTTAGGTCCTTTCCCA-3'. A SalI site was added at the 3' terminus of the fusion product using the primers pcDNA3/F and 5'-CCGGCGGTACACTTTAGGTCCTTTCCCA-3'.

XhoI and XbaI sites were added to the 5' and 3' termini of the downstream fragment respectively, using the primers 5'-ATATATTCTCGAGCCCACACCTGGGAAA-3'and 5'ATATTCTAGATTAGGGCCCAGGCTGGTT-3'. The previous fusion product was then digested with HindIII and SalI, while the downstream fragment was digested with XhoI and XbaI. The two fragments were amplified via TOPO cloning into pCR-BluntII-TOPO (Life Technologies) and fused together via ligation. The full-length sequence of EML4-ALK v1 was then inserted between the HindIII and XbaI sites of pcDNA3.1.

The pcDNA3.1 expression vectors containing the tyrosine kinase-inactive mutants EML4-ALK 3a K315M, EML4-ALK v3b K326M, EML4-ALK v1 K589M, and KIF5B-ALK K1013M were generated by amplifying the whole length of their respective wild-type templates with the 5'-phosphorylated primers 5'-TGACGCTGCCTGAAGTGTGCTCTGAACAGG-3' and 5'-TCACAGCCACTTGCAGGGGGCTTGGGTCGT-3', to convert the lysine codons in the active sites to methionine. The linear mutant plasmids were re-circularized via ligation with T4 DNA ligase (ThermoScientific). All mutations were verified via sequencing.

Cell culture and transfection

HEK293T (human embryonic kidney) cells were cultured in complete medium [DMEM (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Hyclone), 4 mM L-glutamine, 50 U/mL penicillin (Invitrogen) and 100 mg/mL streptomycin (Invitrogen)] at 37 °C in an incubator containing 5% CO_2. For transfections, sub-confluent cells were transfected with GeneJuice transfection reagent (Novagen) as per the manufacturer's instructions.

Drug treatment and western blot analysis

HEK293T cells were seeded in 12-well plates, and at approximately 10% confluency each well was transfected with 0.3 μg of either EML4-ALK v3a, v3b, v1 or KIF5B-ALK plasmid. At 48 hours post-transfection and about 90% confluency, cells were treated with either 2 μM geldanamycin [Hsp90 inhibitor] (AG Scientific), 20μM MG132 [proteasome inhibitor] (Sigma-Aldrich), 1 μM MLN4924 [Nedd88-activating enzyme inhibitor] (Sigma-Aldrich), or co-treated with 2 μM geldanamycin and 20 μM MG132 for 10 hours.

Cells were washed with ice-cold phosphate buffered saline (PBS) and then lysed in Triton X-100 containing lysis buffer [25 mM Tris–HCl (pH 7.5), 100 mM NaCl, 2.5 mM EDTA, 2.5 mM EGTA, 20 mM NaF, 1 mM Na_3VO_4, 20 mM sodium β-glycerophosphate, 10 mM sodium pyrophosphate, 0.5% Triton X-100, Roche protease inhibitor cocktail, 0.1% β-mercaptoethanol]. Whole cell lysates were cleared via centrifugation prior to western blot analysis. Equal amounts of protein were loaded into 6% denaturing SDS-PAGE gels and resolved, and then transferred to nitrocellulose membranes (GE Healthcare). Membranes were probed with rabbit monoclonal anti-ALK antibody (Cell Signaling), as well as mouse monoclonal anti-human β-actin (Sigma-Aldrich) as an internal loading control.

Protease sensitivity assay

HEK293T cells were grown and transfected with 0.3 μg of EML4-ALK v3a, v3b, v1 or KIF5B-ALK plasmid for 48 hours as described in 2.3. Cells were washed in ice-cold PBS and lysed with the same Triton X-100 lysis buffer, but containing 1 mM dithiotreitol (DTT), and without protease inhibitors added. 50 μL (1.5 μg/μL protein) of lysate was subjected to limited trypsin digestion (100 μg/mL) at room temperature, for a 60-min time course. Aliquots were taken every 10 minutes and mixed with 4X SDS sample buffer, then immediately boiled at 100 °C to terminate the reaction. Samples were loaded onto 10% SDS-PAGE gels and analyzed by western blot.

Crosslinking assay

HEK293T cells were grown and transfected with 0.6 μg of pcDNA3.1 empty vector (negative control), EML4-ALK v3a, v3b, v1 or KIF5B-ALK plasmid for 48 hours as described in 2.3. As a positive control, untransfected cells were treated with MG132 for 6 hours, at 48-hours post-transfection, as the drug is reported to induce HSF1 trimerization and activation (Kim & Li, 1999). Cells were washed with ice-cold PBS and lysed in Triton X-100 with lysis buffer [25 mM HEPES (pH 7.4), 100 mM NaCl, 1 mM DTT, 5 mM EDTA, Roche protease inhibitor cocktail, 0.5% Triton X-100]. 100 μL of lysate (1.5 μg/μL protein) was crosslinked with 2 mM ethylene glycol bis[succinimidylsuccinate] (EGS) at room temperature for 30 minutes. The crosslinking reaction was quenched by addition of 20 mM Tris. Samples were immediately boiled at 100 °C in 4X SDS sample buffer, loaded into 6% SDS-PAGE gels and analyzed by western blot. HSF1 trimers were detected using mouse polyclonal anti-HSF1 antibody.

Luciferase reporter assay

HEK293T cells were seeded in 12-well plates and transfected with 0.3 μg of pcDNA3.1 empty vector (negative control), EML4-ALK v3a, v3b, v1 or KIF5B-ALK plasmid, and co-transfected with 0.2 μg of either one of the luciferase reporter vectors Hsp70B/Luc or 3xHSE/Luc (kindly provided by Dr Wulf Paschen, Duke University) for 48 hours. Cell lysis was carried out using the Steady-Glo© Luciferase Assay System (Promega) as per the manufacturer's instructions. Luciferase activity was measured using a 20/20n Luminometer (Turner Biosystems). Data are presented as the average ± SD of a representative experiment performed in duplicates.

Results and Discussion

Folding of ALK fusion oncoproteins is highly dependent on Hsp90 chaperone activity

To determine whether chaperone-dependent folding pathways are important for ALK fusion protein expression, HEK293T cells expressing the EML4-ALK isoforms v3a, v3b, and v1 or KIF5B-ALK were treated with the Hsp90 inhibitor geldanamycin. The variants differ in structure only in their EML4 domains, with EML4-ALK v1 containing the largest proportion of the EML4 gene (including additional WD40 and HELP domains not found in v3a and v3b). Nonetheless, all three variants, along with KIF5B-ALK, express coiled-coil domains in the N-terminal portion that facilitate homodimerization and activation of ALK. The results show that protein levels of all four ALK fusion proteins markedly decreased upon treatment with geldanamycin at 2 μM for 10 hours. This suggests that misfolding due to decreased chaperone function leads to a high rate of degradation (Figure 1).

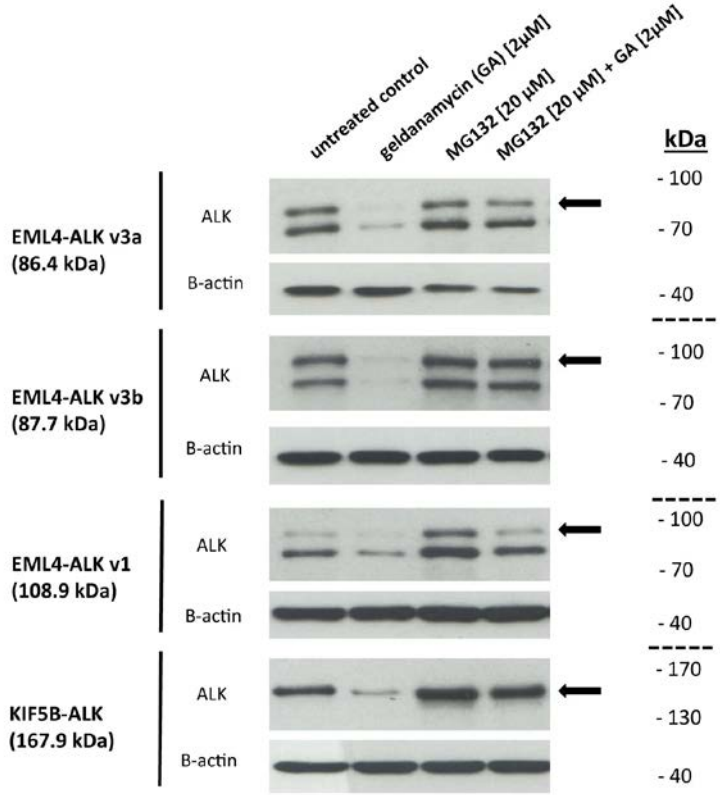

Figure 1. Effect of Hsp90 and proteasome inhibition on ALK fusion protein expression in HEK293T cells. To determine the effect of Hsp90, proteasome and NAE inhibition on ALK oncogene expression, cells were treated exclusively with 2 μM geldanamycin (GA), 20 μM MG132, or co-treated with the same concentrations of GA and MG132 for 10 hours at the specified concentrations. Cell lysate samples were resolved by SDS-PAGE, and total ALK protein levels were measured via western blot analysis. β-actin levels were probed as an internal loading control. Arrows indicate full-length EML4-ALK and KIF5B-ALK protein bands.

All three EML4-ALK variants exhibited similar sensitivity to Hsp90 inhibition, in contrast to a previous study that reported greater susceptibility to degradation for EML4-ALK v1 upon treatment with the geldanamycin analogue 17-DMAG (Heuckmann et al., 2012) in Ba/F3 cells. This discrepancy might be due to the differences in the experimental conditions or cell line specific effects.

To date, the intrinsic stability and susceptibility of the novel ALK fusion protein KIF5B-ALK to degradation and misfolding has yet to be studied. Our results indicate that KIF5B-ALK is highly sensitive to treatment with geldanamycin and undergoes substantial degradation upon Hsp90 inhibition, suggesting that its C-terminal kinesin domain confers it with inherent structural instability it much the same way that the EML4 portion destabilizes variant 1 for EML4-ALK (Figure 1).

To determine the degree to which proteasomal degradation is occurring among the ALK fusion proteins at steady state, HEK293T cells expressing the oncoproteins were treated with MG132 (a peptide aldehyde that inhibits the proteolytic activity of the 26S proteasome complex) at 20 μM for 10 hours. An increase in expression levels (compared to untreated controls) was observed for the longer, intrinsically unstable variants EML4-ALK v1, but not for EML4-ALK v3a or v3b (Figure 1). This confirms previous reports of differential stability among the three EML4-ALK fusion variants (Heuckmann et al., 2012). In addition, KIF5B-ALK also demonstrated a high degree of steady-state degradation, with protein levels increasing upon proteasome inhibition. This corroborates the previous findings on the effect of Hsp90 inhibition on KIFB-ALK expression, suggesting that the oncoprotein is structurally unstable.

To test whether proteasome inhibition could rescue the ALK fusion protein downregulation upon Hsp90 inhibition, transfected cells were co-treated with both geldanamycin and MG132. For all four variants, MG132 treatment restored protein expression to their steady-state levels, suggesting that degradation induced by protein misfolding is mediated primarily by the ubiquitin-mediated 26S proteasome-dependent pathway (Figure 1). However, for EML4-ALK v1 and KIFB-ALK, co-treatment with MG132 did not restore expression levels in Hsp90-inhibited cells to the amount of protein observed with MG132 treatment alone. This suggests that upon increased misfolding (as often observed in tumorigenic cells), protein turnover may be regulated by additional cytosolic degradation pathways independent of the proteasome.

A crucial regulator in the classical ubiquitin-dependent proteasome degradation pathway (the principal mechanism that marks most cytosolic proteins for proteolysis) are E3 ubiquitin ligases, about half of which belong to a subclass known as the cullin-RING ligases (CRLs). This proteolytic route is regulated by the ubiquitin-like protein NEDD8, which is activated and conjugated to CRLs to induce ubiquitin-dependent protein degradation (Petroski & Deshaies, 2005). To test whether degradation of the ALK fusion proteins are CRL substrates dependent on NEDD8 activity, the cells were treated with MLN4924, a small molecule inhibitor of the NEDD8-activating enzyme (NAE). Drug treatment, however, did not result in a substantial increase in ALK protein levels (data not shown). The possible exceptions are EML4-ALK v1 and KIF5B-ALK, which showed only a slight increase in expression, though not to the same extent as proteasome inhibition by MG132 (data not shown). This suggests that the

ubiquitinylation and subsequent degradation of these unstable ALK fusion variants is largely independent of CRL activity.

EML4-ALK v3a, v3b, v1 and KIF5B-ALK are highly sensitive to protease degradation

To investigate the intrinsic susceptibility of the ALK fusion protein to protease degradation due to a more open structural conformation or exposure of normally nested residues, a protease sensitivity assay was performed on all four oncoproteins. This assay is based on the principle that correctly folded proteins have a generally more compact structure that is resistant to degradation by low concentrations of an unspecific protease, whereas unfolded or chaperone-bound proteins are more susceptible to digestion (Stidham, et al., 2003). Trypsin, a serine protease that cleaves peptide chains at the carboxyl ends of lysine or arginine residues, was chosen for this study. To validate the assay, the degradation rates of heat-treated EML4-ALK v3a and KIF5B-ALK (obtained from heated HEK293T whole cell lysates) were compared with untreated samples. As shown in Figure 2A, the degradation of the heat-treated EML4-ALK v3a and KIF5B-ALK was faster (i.e. the high molecular weight protein band densities diminished more quickly) compared to unheated controls.

To characterize the impact of Hsp90 inhibition on the accumulated misfolding of the ALK fusion variants, HEK293T cells expressing the oncoproteins were treated with geldanamycin, and subsequently lysed and subjected to trypsin digestion. Overall, the degradation rates of all four variants were dramatically increased upon Hsp90 inhibition, with full-length EML4-ALK disappearing completely within 10 min (as opposed to about 30-40 min for the untreated controls), and full-length KIF5B-ALK disappearing after 30-40 min (as opposed to >60 min for the control) (Figure 2B). This implies that the native conformations of these oncoproteins are easily disrupted under proteoxic stress that induces misfolding. Possibly, the exposure of normally nested hydrophobic residues increases their dependence on chaperonage by Hsp90 and susceptibility to proteasome detection. In light of the previous drug treatment results, these findings collectively suggest that the folding cycles and turnover of the ALK fusion variants, in particular EML4-ALK v1 and KIF5B-ALK, are highly dependent on Hsp90 chaperone function and proteasome degradation.

KIF5B-ALK oncogene induces limited HSF1 trimerization

It was established in the previous experiments that the ALK fusion variants are intrinsically unstable and highly susceptible to misfolding and degradation when molecular chaperone activity is compromised. Thus, we hypothesized that targeting the principal mediator of heat shock gene transcription, HSF1, would be a suitable therapeutic strategy for decreasing expression levels of ALK oncoproteins. Previous studies have verified that HSF1 is rapidly transactivated in response to various types of proteotoxic stress that induce protein misfolding

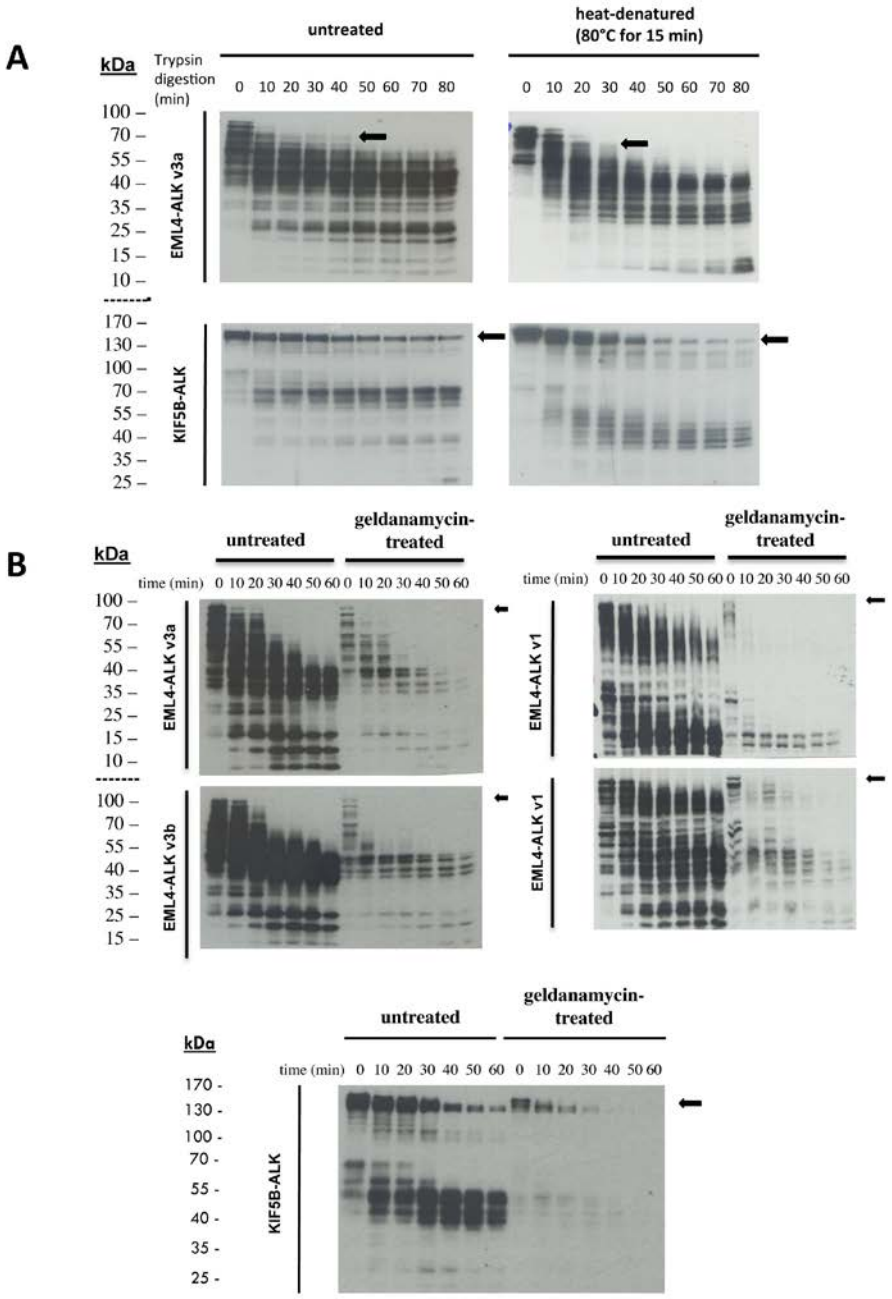

Figure 2. Protease sensitivity assay for ALK fusion proteins. To characterize the stability and propensity of the ALK fusion variants to misfolding, whole cell lysates of HEK293T expressing these oncoproteins were subjected to limited trypsin digestion for the times indicated. ALK protein levels were tracked over the duration of the time course via western blot. Arrows indicate full-length protein bands. (A) To validate the sensitivity of the assay to protein misfolding, the degradation rates of heat-denatured EML4-ALK v3a and KIF5B-ALK were compared to unheated controls. (B) To further induce misfolding, cell lysates were treated with 2 μM geldanamycin for 6 hours, and the trypsin degradation rates for all ALK fusions were compared with untreated controls. Results for EML4-ALK v1 are shown in duplicates.

and, consequently, promote upregulation of the classical chaperones (Hsp27, Hsp70, Hsp90, etc.). In addition to heat shock, hypoxia, ischemia and osmotic stress, expression and activation of HSF1 is particularly sensitive to the over-accumulation of misfolding-prone oncogenic proteins (Hong et al., 2001). Apart from mediating protein folding, the HSF1 transcriptome further supports tumorigenesis by regulating genes related to metabolism, cell cycle signaling, apoptosis and proliferation (Hoang et al., 2000). This heightened "non-oncogene addiction" or dependence on HSF1 thus renders it a promising inhibitory target for preventing tumor formation.

HSF1 usually exists in the cytosol as a monomer with limited DNA-binding activity. Upon increased proteotoxic stress, HSF1 homotrimerizes, becomes hyperphosphorylated and translocates into the nucleus to bind to consensus heat shock element (HSE) sequences upstream of the target Hsp genes, thereby inducing transcription of the Hsp chaperones. To determine whether overexpression of the ALK fusion proteins induces HSF1 trimerization, HEK293T cells were transiently transfected with the four ALK variants, and whole cell lysates were treated with the crosslinking reagent EGS (Figure 3). EGS contains two reactive NHS ester groups, separated by a spacer. After hydrolysis of the NHS ester, the reactive groups form stable amide bonds with primary amine groups on lysine residues of interacting proteins. As a result, protein homomers and heteromers become covalently linked. Interestingly, the results show that there is increased HSF1 trimerization upon overexpression of the unstable fusion variant KIF5B-ALK, compared to the negative control expressing the empty vector. However, the trimerization with KIF5B-ALK was not as dramatic as the MG132-treated positive control. Surprisingly, the three EML4-ALK variants did not appear to induce any increase in HSF1 trimer levels as predicted, suggesting that basal levels of HSF1 trimerization may be sufficient to support the folding cycles of these oncoproteins. The results, confirm, nonetheless, the inherent structural instability of KIF5B-ALK and its striking dependence on Hsp chaperone expression and activity.

Overexpression of EML4-ALK variants and KIF5B-ALK increases HSF1 transcriptional activation independent of constitutive ALK activity

To determine the effect of EML4-ALK and KIF5B-ALK expression on HSF1 transcriptional and DNA-binding activity, HEK293T cells expressing the fusion variants were co-transfected with reporter plasmids that detect HSF1 binding to either the Hsp70B promoter (Hsp70B/Luc) or an idealized HSE consensus sequence (3xHSE/Luc) upstream of the firefly luciferase gene (Figures 4A and 4B).

In contrast to the previous crosslinking results which showed HSF1 trimerization to be induced only by KIF5B-ALK, the luciferase assay results for both reporters suggest that HSF1 transcriptional activity was significantly increased upon expression of all four fusion variants, in comparison to an empty-vector-transfected control. Predictably, the more unstable variants (EML4-ALK v1 and KIF5B-ALK), which depend greatly on Hsp mediation to properly fold, also induced the highest levels of HSF1 transcriptional binding. Thus, there is a direct correlation between the intrinsic structural stability of the ALK fusion variants and the degree to which they activate HSF1.

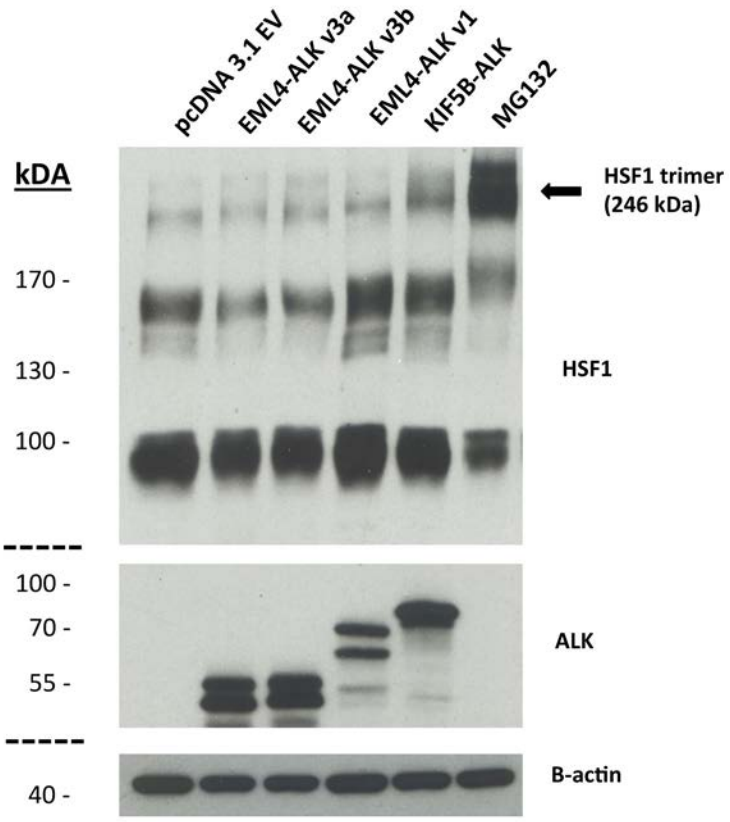

Figure 3. Effect of overexpression of ALK fusion oncoproteins on HSF1 trimerization. To determine whether the ALK oncoprotein intrinsic propensity to misfolding leads to the transactivation and homotrimerization of HSF1, HEK293T cells were transfected with the four ALK fusion variants. Cell lysate samples were cross-linked with EGS, resolved on SDS-PAGE and probed for relative protein levels of HSF1 monomers, dimers and trimers. Cells transfected with pcDNA 3.1 empty vector were used as a negative control, while MG132-treated cells (with an increased protein load, therefore activating HSF1-mediated folding) were used as a positive control.

To verify that the observed increase in transcriptional activity is due primarily to greater cellular demand for chaperone-mediated folding of the ALK fusion proteins, and is independent of the constitutive kinase activity of the ALK domain (which might activate HSF1 transcriptional activity via other mechanisms, such as induction of HSF1 phosphorylation), cells were transfected with kinase-inactive mutants of the fusion proteins and similarly tested for luciferase reporter activity. The results show that, for both reporters, the increase in HSF1 transcriptional activity is indeed reduced upon inactivation of the kinase domains of the ALK fusion variants, suggesting that the increase is partially kinase-dependent. However, the increase is still significant relative to the negative control, affirming that transcriptional activation of HSF1 is due only in part to the intrinsic instability of the ALK oncoproteins.

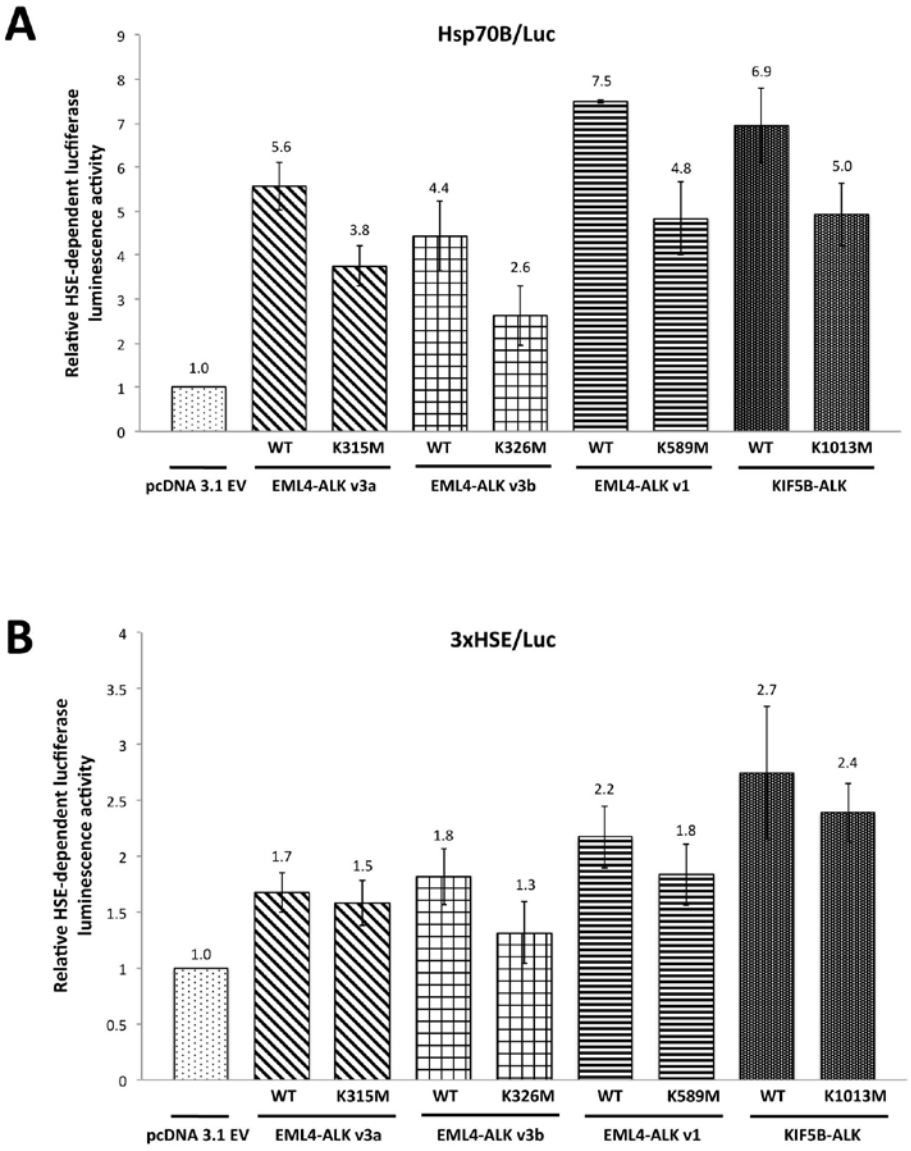

Figure 4. HSE-dependent luciferase reporter assay for cells expressing ALK fusion variants**.** HEK293T cells were transiently transfected with wild-type EML4-ALK v3a, v3b, v1 and KIF5B-ALK, and co-transfected with the luciferase reporter plasmids; **(A)** Hsp70B/Luc; **(B)** 3xHSE/Luc (containing the Hsp70B promoter sequence and three copies of an HSE consensus sequence upstream of the luciferase firefly gene, respectively). To verify that any significant increase in HSE-dependent transcriptional binding is not due to the kinase activity of the ALK domain of the oncoproteins, cells were also transfected with the respective kinase-inactive mutants and luminescence measured. Luciferase activity was measured using the Steady-Glo Luciferase Assay System. Data are presented as fold stimulation relative to control cells transfected with pcDNA3.1 empty vector, with values reflecting the average ± SD of a representative experiment performed in duplicates.

Overexpression of HSF1 does not significantly affect the expression of ALK fusion oncogenes

The foregoing experiments suggest that expressing the EML4-ALK variants and KIF5B-ALK in HEK293T cells leads to a moderate increase in transcriptional activity of HSF1. We therefore hypothesized a dependence of these unstable fusion oncogenes on HSF1-regulated folding. It was thus posited that expression levels of the ALK oncoproteins would be positively correlated with cellular expression levels of HSF1. To test the hypothesis that increasing HSF1 expression might enhance the chaperone-mediated folding efficiency of the ALK fusion proteins, HEK293T cells expressing the variants were co-transfected with either empty pcDNA3.1 vector or pcDNA3.1-FLAG-HSF1 and ALK fusion protein levels measured. However, no change was observed in expression levels for all four fusion variants upon overexpression of HSF1 (Figure 5). This suggests that basal levels of HSF1 are sufficient to maintain stable folding and expression of the various ALK oncoproteins.

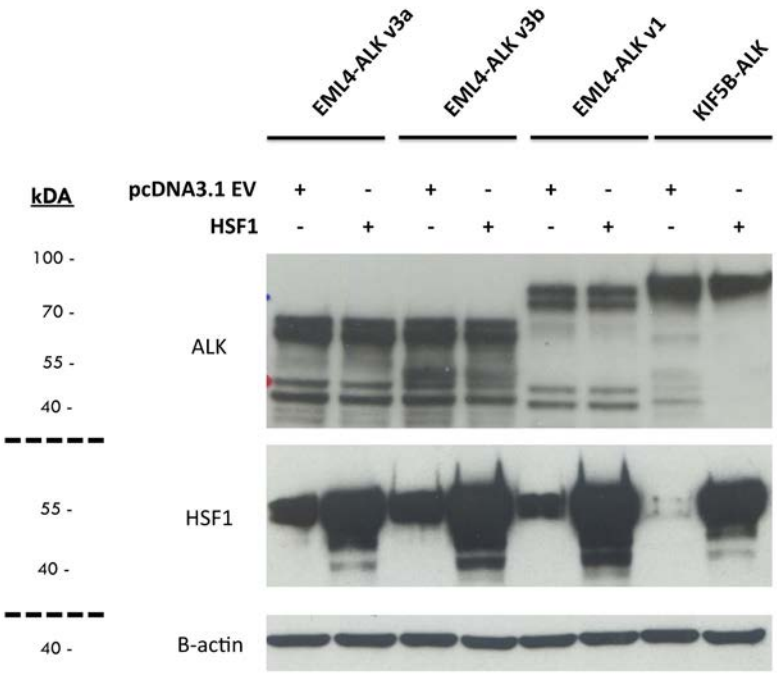

Figure 5. Effect of HSF1 overexpression on ALK fusion protein levels. To determine whether increasing the expression of HSF1 would enhance the folding efficiency of the ALK fusion variants and lead to an increase in their own expression, HEK293T cells were transfected with each of the four ALK oncoproteins and co-transfected with either pcDNA3.1 empty vector (a negative control) or FLAG-HSF1. Lysates were subjected to SDS-PAGE and western blot analysis and probed for ALK and HSF1 protein levels.

Conclusions

In summary, the previous results show that the three EML4-ALK variants exhibit high susceptibility to degradation induced by Hsp90 inhibition. EML4-ALK v1 had the lowest basal levels of expression among the three variants, and was also subject to greater levels of

proteasome degradation, which corroborates previous reports that it is the most structurally unstable. The folding properties of the previously uncharacterized KIF5B-ALK have been shown to be markedly similar to those of EML4-ALK v1, in terms of sensitivity to Hsp90 inhibition and levels of steady state proteasome degradation. Hence, these two ALK fusions are highly unstable and likely to be more responsive to therapeutic strategies targeting their folding cycles. In addition, it has been shown that the turnover rate of these two oncoproteins may be regulated by proteasome-independent pathways. All four oncoproteins were shown to be particularly sensitive to protease digestion, with the degradation effect more pronounced upon heating or inhibition of Hsp90-mediated folding. This provides some evidence that the fusion oncoproteins do indeed assume a more open native conformation that exposes hydrophobic residues and possibly increases their dependence on chaperone activity for stabilization and folding. The above findings suggest that chaperone-assisted folding mechanisms present a viable therapeutic target. However, it was also found that overexpressing the master transcriptional regulator of heat shock protein expression, HSF1, in HEK293T cells appears to have no enhancing effect on ALK oncoprotein levels. This suggests that HSF1-directed strategies may have limited therapeutic effectiveness. Promisingly, however, the expression of KIF5B-ALK in mammalian cells has been shown to induce HSF1 trimerization and activation, while all four ALK fusion proteins have been shown to increase HSF1 transcriptional activity independent of their constitutive kinase function. This suggests that HSF1 is still greatly implicated in their folding cycles and stabilization, though it may prove to be too indirect a target for inducing degradation and substantially attenuating protein levels of the ALK fusion oncokinases. Further studies could be potentially done to determine if inhibition of HSF1 expression would have synergistic therapeutic effects when combined with other ALK-directed treatments, so as to take full advantage of its strategic role in promoting oncogenesis.

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