IN THE SPOTLIGHT

EML4–ALK Fusions: Propelling Cancer but Creating Exploitable Chaperone Dependence

Paul Workman and Rob van Montfort

Summary: The crystal structure of a conserved tubulin-binding region of the EML1 protein reveals a highly atypical fold in one of its β-propeller domains. Disruption of the EML1 core region domain in many of the oncogenic EML4–ALK fusion protein variants that drive non–small cell lung cancer explains their dependence on the HSP90 molecular chaperone, provides a basis to allow more precise patient stratification for therapy, and suggests a more general model for other oncogenic fusion proteins. *Cancer Discov; 4(6); 642–5. ©2014 AACR.*

Echinoderm microtubule-associated protein (EMAP)–like or EML proteins usually function in the cytoskeleton, contributing to the formation of the mitotic spindle and the interphase microtubule network. However, in around 5% of non–small cell lung cancers (NSCLC), chromosome rearrangements generate oncogenic fusion proteins that link parts of EML4 to the anaplastic lymphoma kinase (ALK; ref. 1). EML4–ALK proteins are highly transforming and pathogenic in NSCLC due to increased oligomerization and constitutive, kinase-activating autophosphorylation. However, at the same time, these fusion proteins create the therapeutic Achilles heel of an oncogenic addiction state in which the cancer cells depend on the tyrosine kinase activity of the fusion protein for survival—thus explaining the impressive clinical responses to crizotinib and other small-molecule drugs inhibiting the ALK tyrosine kinase (2). Unfortunately, despite the substantial clinical benefit, resistance to crizotinib is very common, frequently arising through secondary mutations in the ALK kinase component of the oncogenic driver fusion protein (3).

Encouragingly, studies in EML4–ALK–positive human NSCLC cells *in vitro* and in corresponding animal models show that it is possible to overcome various molecular forms of resistance to ALK inhibitors, including the common secondary ALK mutations, by treatment with small-molecule inhibitors of the molecular chaperone heat shock protein 90 or HSP90 (3, 4). These drugs are currently emerging as remarkably versatile molecular therapeutics, based on the unusually high level of molecular dependence of many oncogenic kinases and ligand-dependent transcription factors on chaperoning by HSP90 to maintain oncoprotein stability and function (5). Inhibition of HSP90 leads to loss of signaling output and proteasomal degradation of oncoprotein "clients" of the chaperone, including EML4–ALK.

Furthermore, clinical activity has been confirmed with HSP90 inhibitors in patients with EML4–ALK–positive NSCLC whose tumors are resistant to crizotinib (6). However, there are many different EML4–ALK fusion variants, and not all patients with EML4–ALK fusions respond to HSP90 inhibitors.

Hence, the scene was set to look for a convincing molecular explanation—and ideally a structural basis—for the promising and intriguing findings obtained to date linking EML4–ALK fusions to a strong molecular dependence on HSP90 and as a result to therapeutic sensitivity to inhibitors of this chaperone.

Now, in an article published in the *Proceedings of the National Academy of Sciences of the United States of America*, Richards and colleagues (7) have used X-ray crystallography to determine the molecular structure of the conserved, tubulin-binding region of EML1. This structure reveals an unusual protein fold within a key globular domain. In addition, and most importantly, the research indicates that this globular domain is truncated in approximately 70% of the various different EML4–ALK fusions that are found in patients and provides very convincing evidence that the occurrence of this disruption accounts for the sensitivity to HSP90 inhibition of those particular variants. In contrast, other EML4–ALK variants that completely lack the entire domain are resistant to HSP90 inhibition.

As well as providing a satisfying molecular and structural explanation for HSP90 chaperone dependence of certain EML4–ALK variants, the new findings provide a potential basis upon which to predict and identify those particular EML4–ALK fusion–positive patients who will benefit from stratified treatment with HSP90 inhibitors. In addition, the results further suggest that the partial truncation of a globular domain located at the translocation break point may prove more generally predictive of HSP90 inhibitor sensitivity in cancers that are driven by other fusion oncogenes. Furthermore, the new findings shed valuable additional light on the holy grail of HSP90 chaperone biology—the elucidation of the molecular and structural basis underpinning what makes a protein dependent on HSP90 chaperoning.

The new story begins with the determination by Richards and colleagues (7) of the crystal structure of the 70-kDa core

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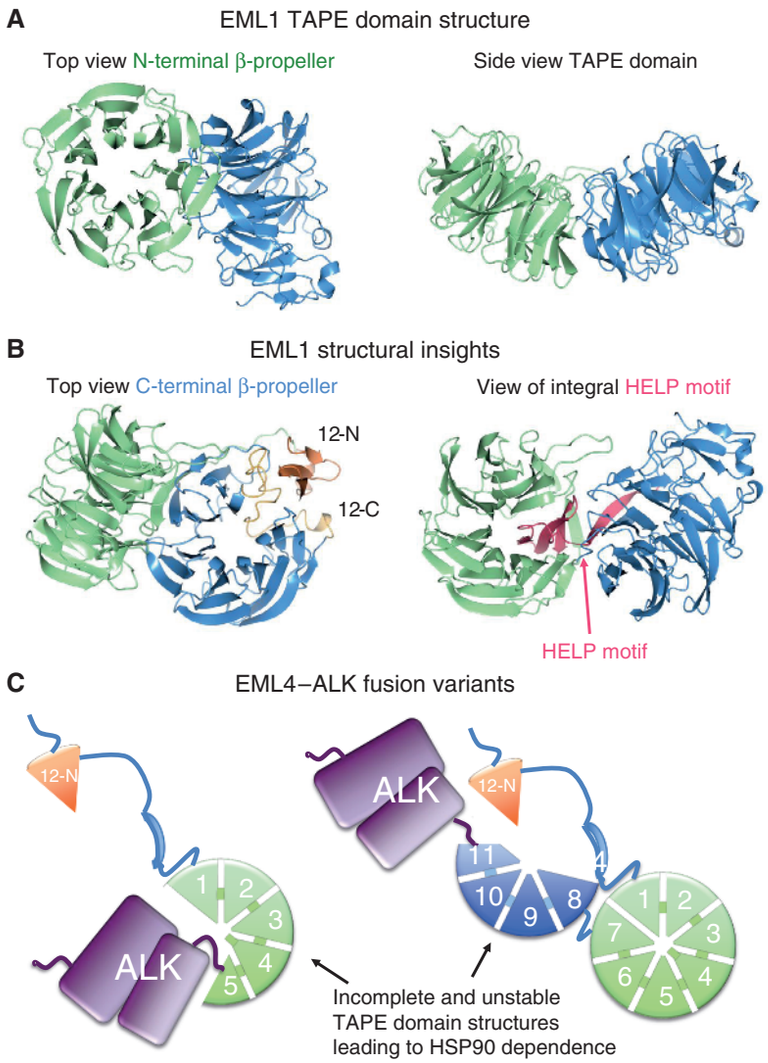

Figure 1. Structure of EML1 and domain organization of oncogenic EML4–ALK fusion variants, as determined by Richards et al. (7). **A,** left, top view of the structure of the EML1 tandem atypical propeller in EMLs (TAPE) domain, which is composed of two seven-bladed β-propeller domains (PDB code 4ci8). The N-terminal propeller is shown in green, the C-terminal propeller in blue. **A,** right, side view of the TAPE domain. The β-propeller domains are positioned at an angle of 50° with respect to each other, creating concave and convex domain surfaces. **B,** left, top view of the C-terminal propeller domain with an unusual 12th blade, which is composed of an N-terminal subdomain (12-N, orange) and C-terminal subdomain (12-C, beige) from distant parts of the primary amino acid sequence. **B,** right, view of the hydrophobic EML protein (HELP) motif (pink), which is an integral part of the TAPE domain and has a mainly structural role in stabilizing the β-propellers and the interface between the two propeller domains. **C,** domain organization of NSCLC EML4–ALK fusion proteins variant 1 (left) and 2 (right). In light of the structure of the EML1 TAPE domain, it can be inferred that these oncogenic fusion proteins have incomplete TAPE domains, which renders them structurally unstable and hence more dependent on the molecular chaperone HSP90. This prediction was confirmed experimentally in cell experiments in which reduced signaling output and degradation of EML4–ALK proteins was observed following HSP90 inhibition for predicted disrupted but not nondisrupted variants.

of EML1. This revealed two tightly interconnected domains—significantly, both with a so-called "β-propeller" fold—that are positioned at an angle of 50° with respect to each other (Fig. 1A and B). Overall, β-propeller protein domains are highly symmetrical disk-like structures, generally composed of four to eight antiparallel β-sheet modules, referred to as the "blades" of the propeller, distributed around a central conically shaped tunnel (8). This circular arrangement of β-sheet modules provides for a fairly rigid and stable platform with loops protruding from the top and bottom, enabling the impressive functional diversity that is observed for β-propeller proteins (8).

Each of the β-propeller domains in the EML1 structure has seven blades. These blades are numbered consecutively as 1 to 7 in the N-terminal propeller and 8 to 14 in the C-terminal propeller (Fig. 1C). The N-terminal β-propeller has classical features, with the exception that the seventh and final blade consists of a continuous string of amino acid residues—instead of the more frequently observed arrangement in which the last blade comprises three strands of which the fourth strand is supplied by an N-terminal part of the sequence that closes and stabilizes the propeller fold in a manner often described as "molecular Velcro."

One of the main surprises of the new EML1 crystal structure is the atypical fold of the second, C-terminal propeller, which has one blade (number 12) that does not have the typical four-stranded β-sheet topology, but rather is composed of two subdomains provided from distant parts of the primary sequence (Fig. 1B). One of these, the N-terminal subdomain known as blade 12-N, forms a three-stranded β-sheet, whereas the other, the C-terminal subdomain referred to as blade 12-C, adopts a loop conformation that lacks significant secondary structure. This novel and uncharacteristic propeller blade configuration adds further architectural diversity to the family of β-propeller fold structures and suggests that other noncanonical blade configurations are still waiting to be discovered. Because of its very atypical arrangement, Richards and colleagues (7) named the EML1 core structure a tandem atypical propeller in EMLs (TAPE) domain.

Interestingly, the new EML1 structure also sheds important light on the role of the so-called conserved hydrophobic EML protein (HELP) motif, previously thought to form a distinct domain involved in interactions with microtubules. However, the crystal structure now clearly reveals that the

HELP motif is not a separate domain, but is in fact an integral part of the hydrophobic core of the tandem propeller structure, located at the interface between the two β-propeller domains (Fig. 1B). Of note, deletion of the HELP motif would clearly have a detrimental effect on the stability and folding of the TAPE domain, and the elucidation of its location within the EML1 structure should therefore aid the design of follow-up functional studies.

Having shown that the HELP motif has a mainly structural role, Richards and colleagues (7) next set out to investigate the EML–tubulin interaction; this was triggered by the authors' observation that during purification of EML1 TAPE domains expressed in insect cells, the proteins copurified with α/β-tubulin. By mapping the amino acid conservation of surface residues onto the EML1 structure, they identified a conserved region on the concave side of the TAPE domain that could mediate tubulin-binding interactions (Fig. 1A, right). Site-directed mutagenesis of seven residues within this region resulted in a correctly folded EML1 mutant that was unable to bind soluble tubulin—confirming the importance of the concave surface region of the TAPE domain for tubulin interactions.

Next, Richards and colleagues (7) used their EML1 TAPE domain crystal structure to model the impact of various forms of EML1–ALK fusions that include different elements of the EML structure. Typically, oncogenic fusion proteins involve break points in disordered regions of each of the two individual partner proteins (9). With EML1–ALK fusion proteins, Richards and colleagues (7) made the crucial discovery that several of these variants have a high probability of disrupting the globular TAPE domain. As a result of perturbation of the tight interactions within the propeller structure and the resulting exposure of an incomplete hydrophobic core, such EML1–ALK fusion proteins will be significantly less stable than other variants that retain an intact TAPE domain. Hence, they are much more likely to depend on molecular chaperones like HSP90 to maintain their stability.

On the basis of this structure perturbation model, Richards and colleagues (7) then proceeded to investigate in detail four different oncogenic EML4–ALK fusion proteins. Two of these have break points in either the N-terminal EML β-propeller (EML4–ALK variant 1) or the C-terminal β-propeller (EML4–ALK variant 2), both of which would be expected to be structurally unstable and were therefore predicted to be more dependent on HSP90 (Fig. 1C). These were compared with two further fusion proteins (EML4–ALK variants 3a and 5a) that do not contain any core part of the TAPE domain, and which therefore would be predicted to be less structurally unstable and hence less dependent on chaperone stabilization.

Consistent with this prediction, when wild-type mouse embryo fibroblasts were transfected transiently with the individual fusion variants and then treated with HSP90 inhibitor, significant degradation was seen with EML4–ALK variants 1 and 2, for which structural disruption was postulated, whereas degradation was not observed with variant 3a or 5a, for which architectural perturbation was not anticipated. In addition, Richards and colleagues (7) used another, well-defined model system in which EML4–ALK variants 1 and 2 were stably expressed in Ba/F3 murine hematopoietic cells to create lines that were consequently independent of interleukin 3 but instead had become ALK-dependent. Once again, HSP90 inhibition caused fusion protein depletion for these predicted unstable variants, together with inhibition of downstream ERK phosphorylation as a measure of functional signaling output from the ALK fusions. In contrast, degradation and signaling inhibition were not seen with variants 3a and 5a, which were predicted as structurally stable. Furthermore, the complete degradation of EML4–ALK that was observed for the likely structurally disrupted fusion protein variant 1 was accompanied by apoptotic cell death, whereas these effects were not seen with the expected structurally stable variant 3a.

Finally, Richards and colleagues (7) compared HSP90 inhibitor effects in two patient-derived NSCLC cell lines, one harboring endogenous EML4–ALK variant 1 and the other with EML4–ALK 3b. Consistent with structural predictions and the above results in transfected cell models, HSP90 inhibition caused proteasome-dependent degradation of variant 1 that is predicted to be structurally disrupted, along with corresponding inhibition of downstream ERK phosphorylation; whereas, in contrast, no equivalent effects were seen with the anticipated structurally stable variant 3b.

Taken together, the cellular results are clearly consistent with the model in which the EML4–ALK variants with structurally disrupted EML propeller domains are much more dependent on the HSP90 molecular chaperone for their stability and function than are their nondisrupted fusion protein counterparts. On the basis of their findings, Richards and colleagues (7) propose that patients with NSCLC harboring EML4–ALK variants with partially truncated TAPE domains (as well as the EML1–ABL1 fusion, which is pathogenic in T-cell acute lymphoblastic leukemia) will be sensitive to HSP90 inhibitors, whereas, by implication, cancers in which the EML4–ALK fusion driver lacks disruption will be less responsive or resistant.

Current stratification of patients with NSCLC for ALK inhibitor treatment is based on the detection of *ALK* rearrangement by a fluorescence *in situ* hybridization assay. This companion molecular diagnostic method does not discriminate between particular *EML4–ALK* fusions. If the current findings of Richards and colleagues (7) are confirmed in further preclinical studies and if particular fusions prove to be predictive of clinical outcome, then future patient stratification of patients with NSCLC for treatment with HSP90 inhibitors would require determination of the variant-specific genotype. This important possibility now requires prospective evaluation.

In summary, from a cancer therapy perspective, the most important finding from the new work is that the EML1 crystal structure—with its unusual twin β-propeller architecture—reveals exciting insights into the likely structural viability of oncogenic EML4–ALK fusion proteins and provides a satisfying explanation for the molecular basis of the dependence of particular EML4–ALK fusions upon chaperoning by HSP90, suggesting a more sophisticated basis for patient selection. Thus, EML4–ALK fusions propel cancer but create an exploitable chaperone dependence and therapeutic vulnerability.

Additional studies are now needed to confirm and extend the emerging story. It is important to enlarge the comparative genotype–phenotype correlations to include a much larger number of human NSCLC cell lines—and also tumor xenograft models—that harbor the various different *EML4–ALK* genotypes. It is noteworthy that the 50% cell growth inhibitory values for the HSP90 inhibitor ganetespib were 11 and 35 nmol/L in the

two NSCLC cell lines harboring EML4–ALK variants 1 and 3a, respectively. Hence, although the difference is clear, the cell line with nondisrupted variant 3a is only 3-fold less sensitive than that for the disrupted variant 1, suggesting that the remaining sensitivity for variant 3a may be mediated by effects on alternative HSP90 client proteins that contribute to oncogenesis. Note also that patients with NSCLC without *ALK* fusions or *EGFR* mutations can be responsive to HSP90 inhibitors (6).

More mechanistically, it would be valuable to complete the molecular audit trail between *EML4–ALK* fusion gene sequence, structural disruption, protein stability, and HSP90 chaperone dependence by first determining experimentally the actual protein stability of the various EML4–ALK fusions, for example, by thermal denaturation, and then correlating these measurements quantitatively with HSP90 dependence and cellular sensitivity.

Viewing the significance more broadly, the new results suggest the important possibility that structural disruption of the globular domain present at the translocation break point may be more generally predictive of response to HSP90 inhibitors for the large number cancers of various types that are driven by fusion oncoproteins.

Finally, the new findings provide further evidence toward elucidating the poorly understood molecular and structural basis for what makes particular client proteins, for example, kinases, dependent on the HSP90 chaperone. A previous systematic and quantitative study suggested that whereas the HSP90 co-chaperone CDC37 helps HSP90 to recognize the kinase client class, the subsequent HSP90–kinase interaction is determined by structural viability that could be measured by thermal instability, most likely related to disordered regions in the protein (10). The present results add further support to the view that recognition of kinases by HSP90 depends on the presence of disordered regions in proteins—as often induced by pathogenic mutations and translocations—thus providing a structural explanation for therapeutic selectivity against cancer cells versus healthy cells.

Dare we hope that EML4–ALK fusions will help propel us toward finding the holy grail of HSP90 chaperone biology—the general rules underlying the molecular basis for HSP90 addiction?

Disclosure of Potential Conflicts of Interest

P. Workman and R. van Montfort are employees of The Institute of Cancer Research, which has a commercial interest in the discovery and development of HSP90 inhibitors and operates a Rewards to Discoverers scheme. P. Workman declares relevant commercial interactions with Vernalis, Novartis, Chroma Therapeutics, Astex Pharmaceuticals, NuEvolution, and Nextech Ventures. R. van Montfort is a former employee of Astex Pharmaceuticals and declares relevant commercial interactions with Astex Pharmaceuticals and NuEvolution.

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