INVESTIGATION INTO THE ALLOSTERIC REGULATION OF MITOTIC KINESIN EG5

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Introduction

Eg5, the human kinesin-5 motor protein, is required for formation of the mitotic spindle which is essential for the completion of mitosis. The full length monomer contains a ~42 kD N-terminal motor domain that binds both ATP and microtubules. Functioning as a homotetramer (**Fig 1**), Eg5 binds antiparallel MTs and slides them apart via ATP-powered stepping toward the plus-ends of the MTs. This action is required for establishing a bipolar spindle and for mitotic progression (**Fig 2**). Inhibiting Eg5 leads to mitotic arrest and cell apoptosis. Thus, these proteins are attractive drug targets in antitumor therapy. Current antimitotic chemotherapeutic drugs target the spindle MTs, interfering with spindle dynamics. While effective, they have various side effects, including neurotoxicity.

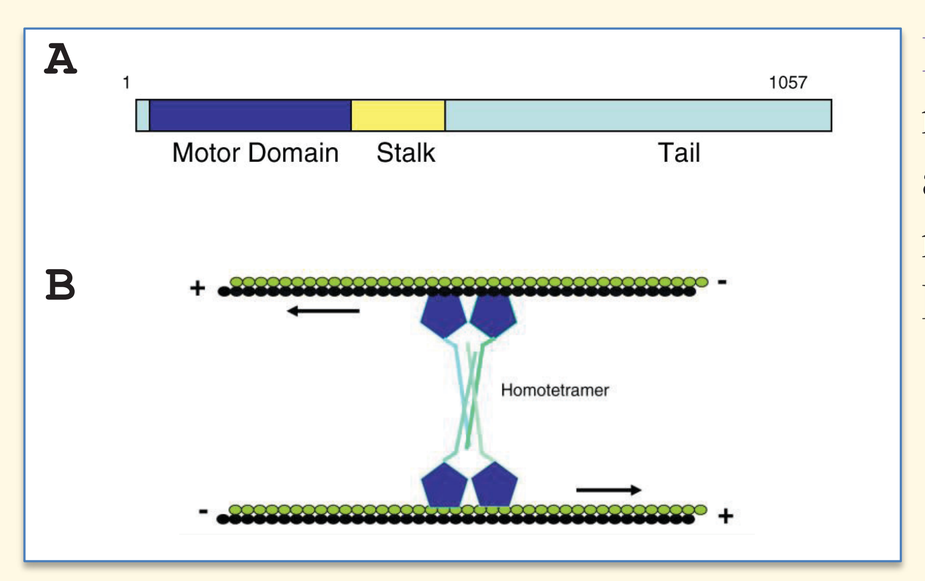


Figure 1 Model of Eg5. (**A**) Schematic of the Eg5 motor. (**B**) The tetrameric structure crosslinks antiparallel MTs; the simultaneous plus-end directed movement along both MTs results in sliding of the MTs apart.

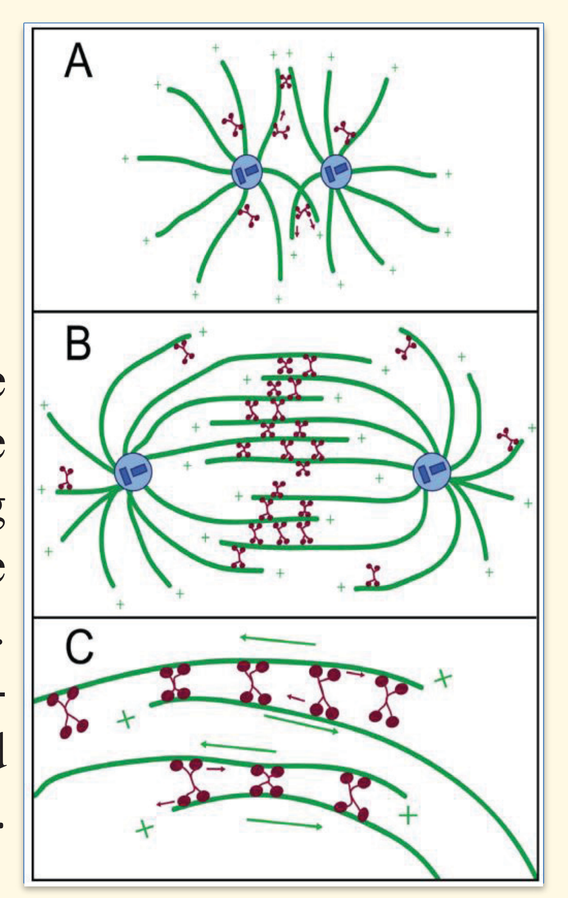


Figure 2 Eg5 activity in the mitotic spindle. (**A**) At the onset of mitosis, Eg5 motors (*red*) may translocate to the plus-ends of MTs, and promote bipolarity by crosslinking antiparallel MTs. (**B**) By metaphase, a stable bipolar spindle has formed. Eg5 motors slide MTs toward the centrosomes. (**C**) Close-up depiction of Eg5 motors walking to the plus-ends of antiparallel MTs, moving both poleward simultaneously..

Allosteric Eg5 inhibitors, like S-trityl-L-cysteine (STC) and monastrol, have created an opportunity for the development of more selective antimitotic chemotherapeutic drugs with an improved side effect profile. These inhibitors interact with the Eg5 motor domain at its surface via the L5pocket, formed by helices α2 and α3 and the L5 loop (**Fig 3**). STC and monastrol are known to prevent ADP release from the motor domain, but the exact mechanism of intramolecular communication between the three ligand-binding sites (ATP-, MT-, and drug-binding site) is not understood. However, recent data show the L5 loop is responsible for allosteric drug binding, and the mechanism for allosteric inhibition is dependent upon the amino acid residues in the L5 pocket.

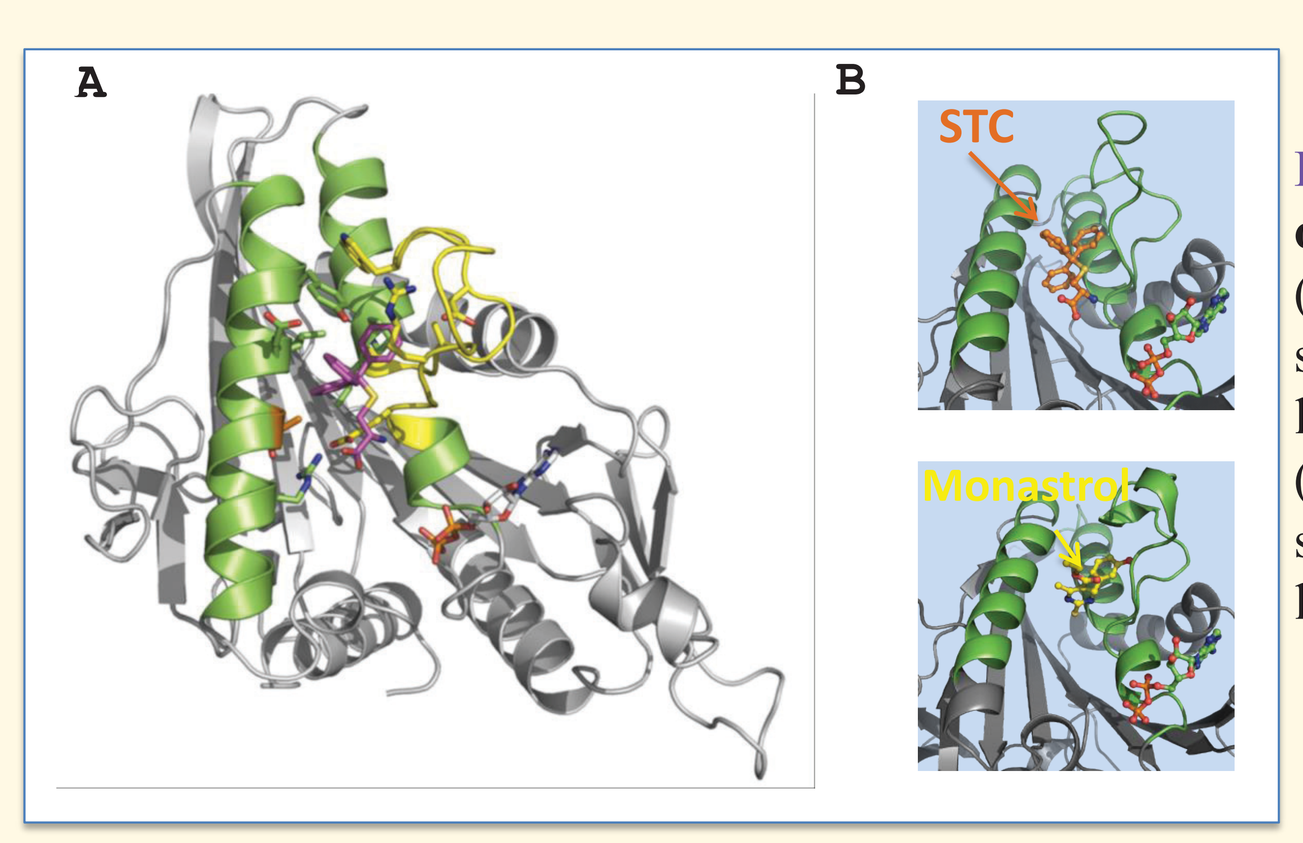


Figure 3 Overview of the location of the L5 pocket. (**A**) STC (*magenta*) surrounded by a schematic view of Eg5 with the L5 loop (*yellow*) and helices α2 and α3 (*green*). (**B**) STC & monastrol surrounded by Eg5 with the L5 loop.

Klp61F, the *Drosophila* homolog to human Eg5, has the same function, a motor domain that is 60% identical, and a similar size L5 loop (**Fig 4**), but Klp61F is not inhibited by either STC or monastrol. Consistent with this lack of inhibition, Klp61F doesn't bind either allosteric effector. However, drug sensitivity can be engineered into the Klp61F motor by replacing its native L5 loop residues with the residues from the human L5 loop.

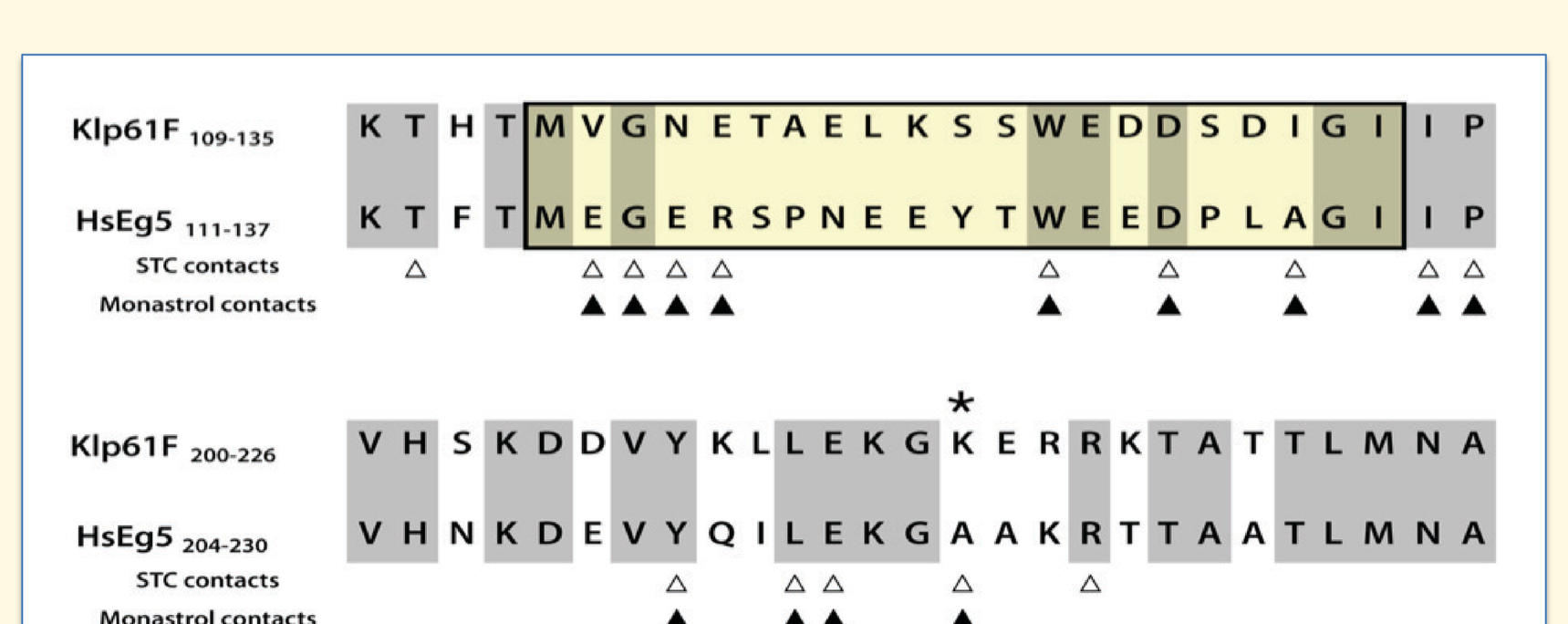


Figure 4 Alignment & spatial location of residues composing the L5 binding pocket of Eg5 to Klp61F. Boxed residues shaded in *yellow* mark the L5 loop. STC contacts with Eg5 differ from those seen in monastrol-bound crystal structures.

Δ – residues within Eg5 in close contact to STC

▲ – residues within Eg5 in close contact to monastrol.

PREVIOUS RESULTS:

The human L5 loop conferred "druggability", resulted in long-distance allosteric inhibition.

Substituting the human L5 loop reconstitutes effector binding to the L5 pocket. The Klp61F chimeria (*Klp61F-L5*) binds both effectors. Similar to Eg5, the Klp61F-L5 is strongly inhibited by STC. Yet, despite binding monastrol, it isn't inhibited by monastrol.

– Basal ATPase rates are inhibited by STC but not by monastrol (**Fig 5**).

– MT-stimulated ATPase rates are inhibited by both STC and monastrol (**Fig 6**).

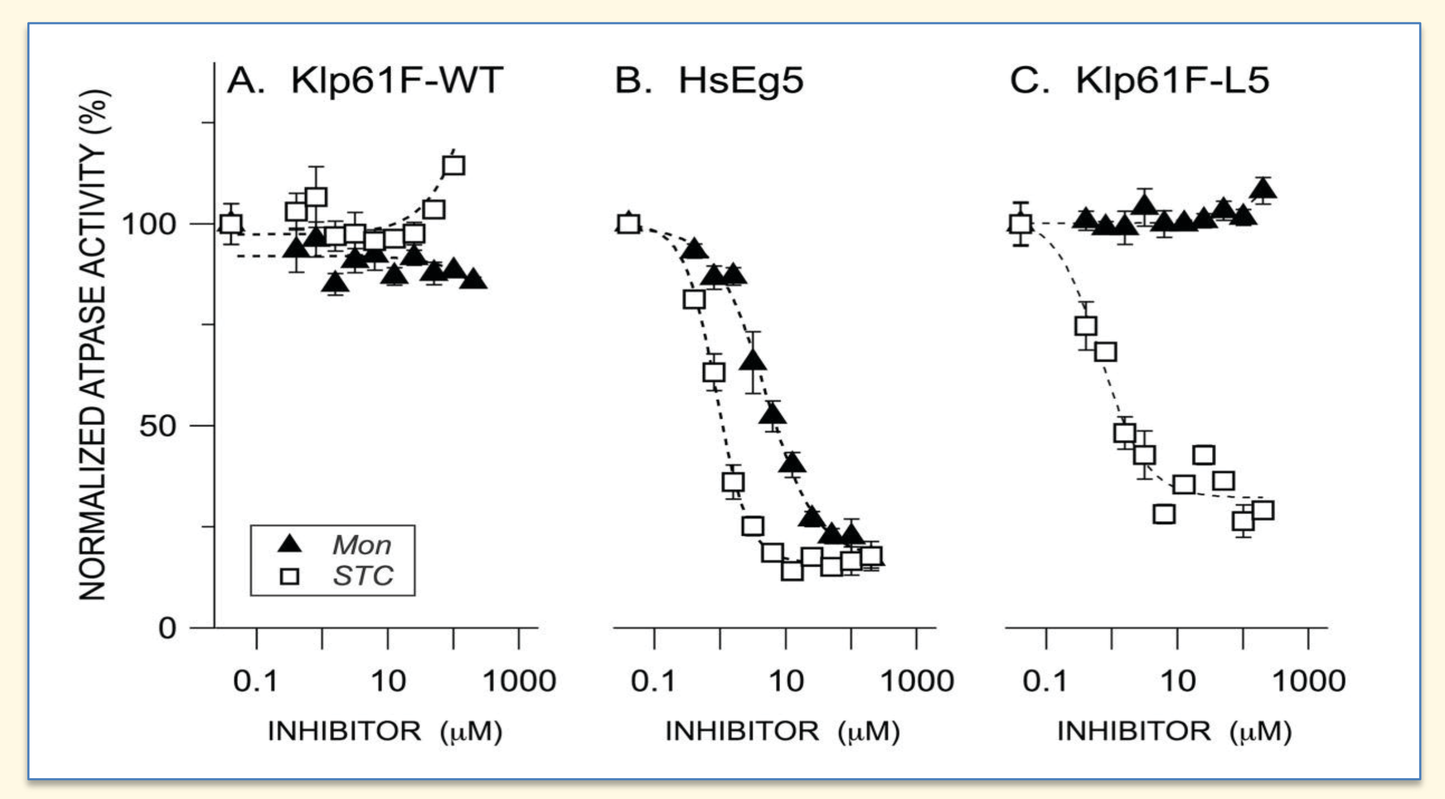


Figure 5 Basal ATPase rates (ADP/motor/sec) measured in the presence of either STC (□) or monastrol (▲).

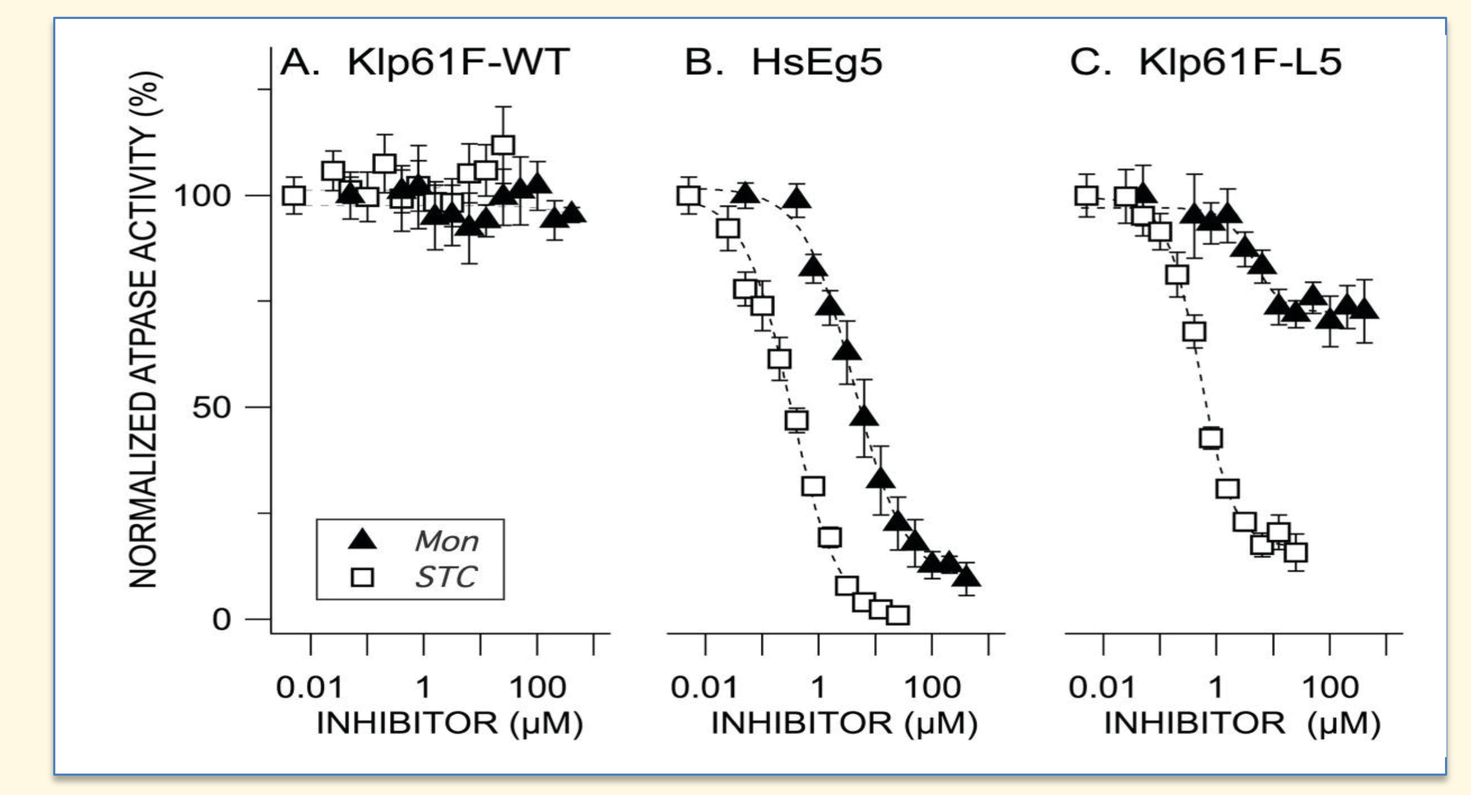


Figure 6 MT-stimulated ATPase rates (ADP/motor/sec) measured in the presence of either STC (□) or monastrol (▲).

More than one allosteric communication network.

Different allosteric inhibitors resulted in different inhibitory effects on KLP61F-L5 chimera. The two effectors may affect inhibition by operating through different allosteric communication networks. These different pathways for eliciting an inhibitory response are likely contingent upon the different modes of contact to the L5 pocket.

The pathway of allosteric inhibition is conserved.

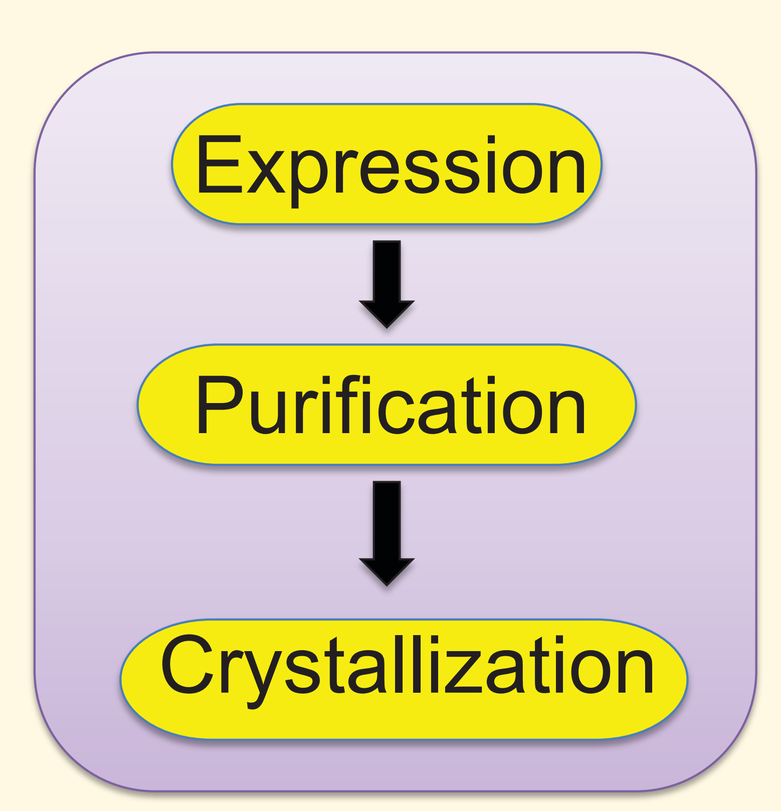
The long distance allosteric network observed originally in Eg5 is conserved in Klp61F. The networks of amino acid residues involved in allosteric communication between the L5 loop and the other two sites (ATP-, & MT-binding sites) may also be conserved across other kinesin family members. Conserved site-to-site communication is therapeutically important. Allostery is a key consideration in drug design.

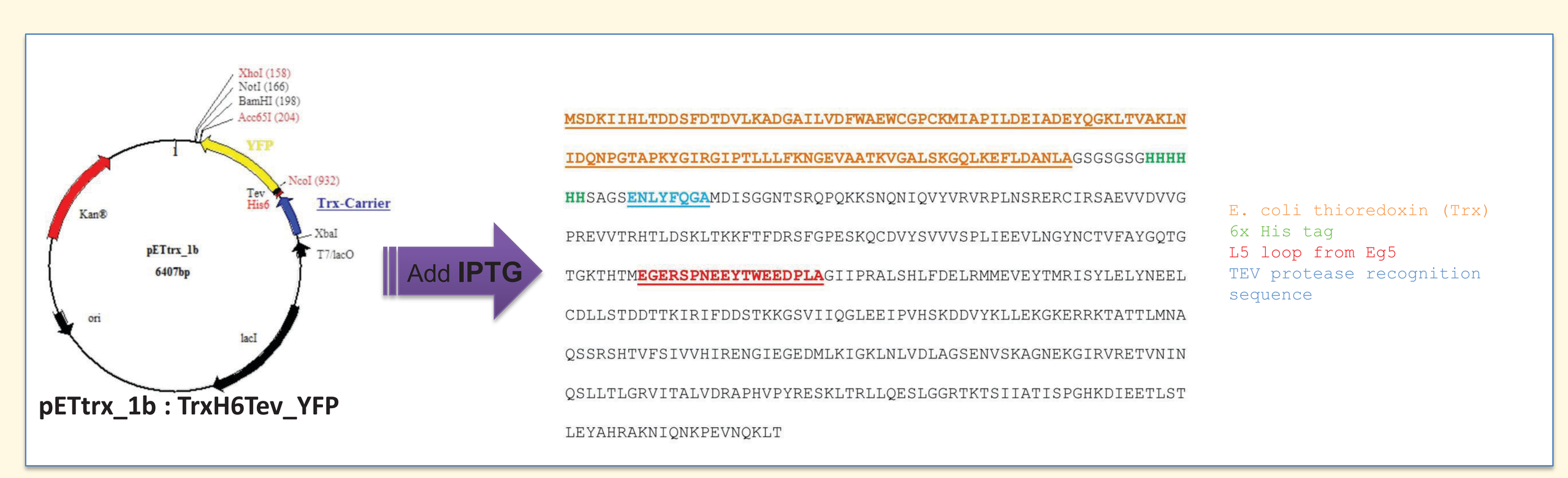
We suspect effector binding elicits parallel structural changes in Klp61F-L5 as those that occur in human Eg5. However, answering these questions is difficult as there are no reported crystal structures of the Klp61F motor domain for comparison studies. The atomic level contacts that mediate allosteric inhibition of Eg5 have not been identified.

Therefore, we sought to determine the crystal structure of the Klp61F-L5 chimera motor domain alone and in complex with STC. Analysis of these crystal structures may provide insight into the nature of the Eg5 allosteric networks and the mechanism of allosteric inhibition by drugs that bind the L5 pocket.

Methods

Expression constructs were transformed with a modified pET vector (*below*) into the Rosetta host strain, and the cells were grown at 27°C to an OD600 of ~0.8. Hoping to improve upon purification from our previous experiments, we decreased the temperature to 18°C before inducing with isopropyl 1-thio-β-D-galactopyranoside (IPTG).





Cells were harvested by centrifugation and lysed using an Emulsiflex. Cell debris was removed by centrifugation, and the supernatant (SN) was applied to a Nickel affinity chromatography column and connected to a Fast Protein Liquid Chromatography (FPLC) system.

After eluting the target protein, the protein solution was digested with Tobacco etch virus (TEV) protease before again being applied onto the Ni column. The His tag-free protein was obtained in the flow through (FT), and loaded onto a S100 gel filtration column.

Pooled fractions containing our target protein were concentrated for crystallization trials. Samples from throughout the purification process were subjected to SDS-PAGE on %12 gels to identify purified protein (**Fig 7, 8, 9**).

Based on prior successful experiments, concentrated chimera protein was mixed with 1mM STC, and crystallization trials were performed by the hanging drop vapor diffusion method and the batch method. Crystals were obtained in several solutions, most of 30-40% PEG 1000 with a 100mM buffer pH 7.5-9 and a 100mM salt. All trials were carried out at room temperature (22°C).

Results

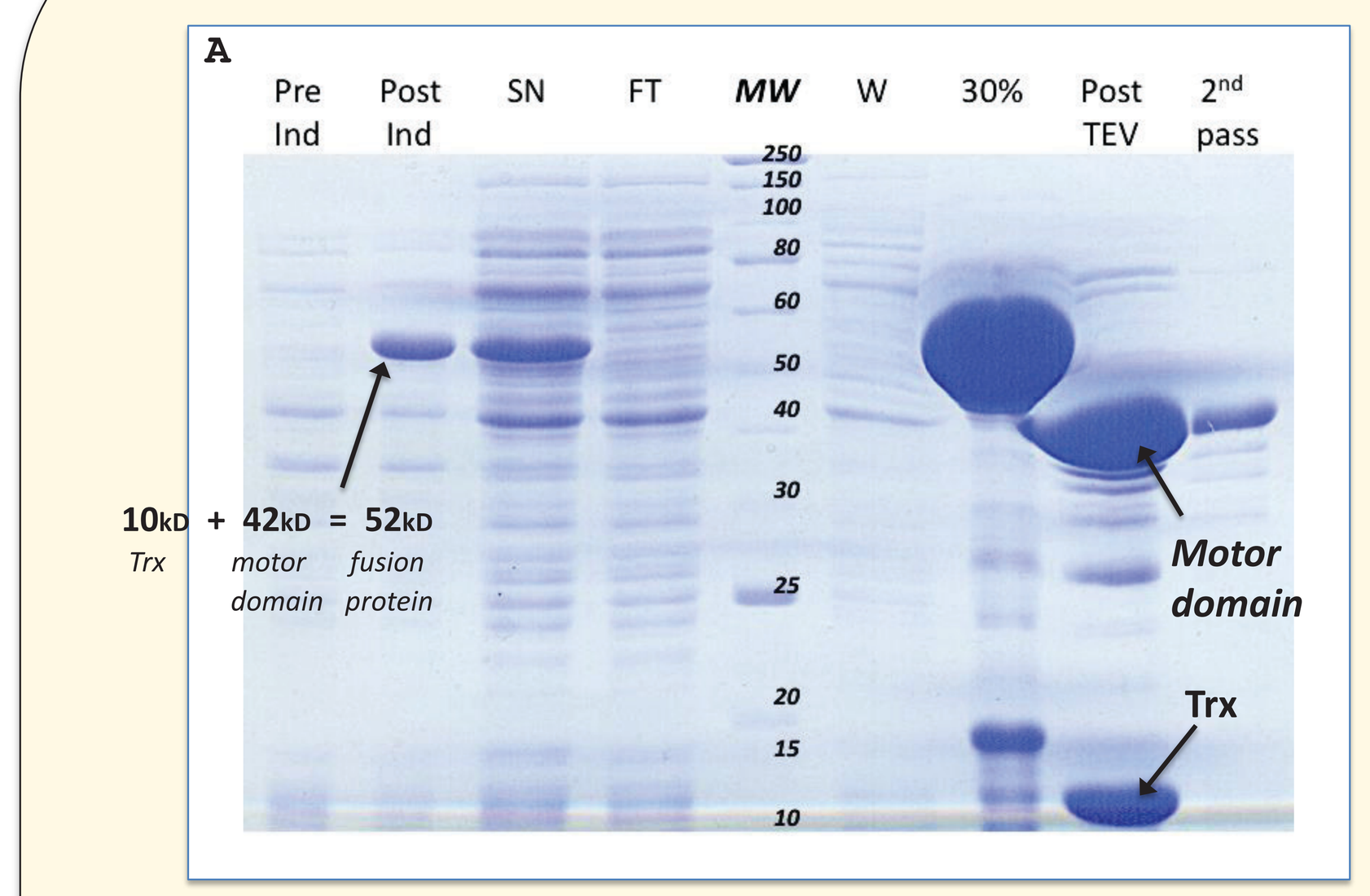


Figure 7 Klp61F-L5 chimera protein purification gel.

Effective expression of the Klp61F-L5 chimera shown with samples taken before induction (Pre Ind) and after induction (Post Ind) with IPTG. 42 kD (Klp61F/Eg5 motor domain) + 10 kD (Trx) = 52 kD (fusion protein, *arrow*).

- Applying the supernatant (SN) to the Ni affinity column, the disappearance of the 52 kD band in the flow-through (FT) indicates fusion protein binding to the column. The FT contains everything that did not bind to the Ni column.
- No fusion protein was lost after washing the column in buffer A (W).
- The fusion protein was eluted using 30% buffer B (30%), seen as the prominent 52 kD band.
- Effective digestion with TEV protease (Post-TEV) is indicated by the band "shift". The Trx (10 kD) was cleaved from the Klp61F chimera motor domain (42 kD).
- Further purification was achieved through a second pass over the Ni column (2nd-pass).

Lower induction temperatures may yield cleaner target protein needed for effective crystallization.

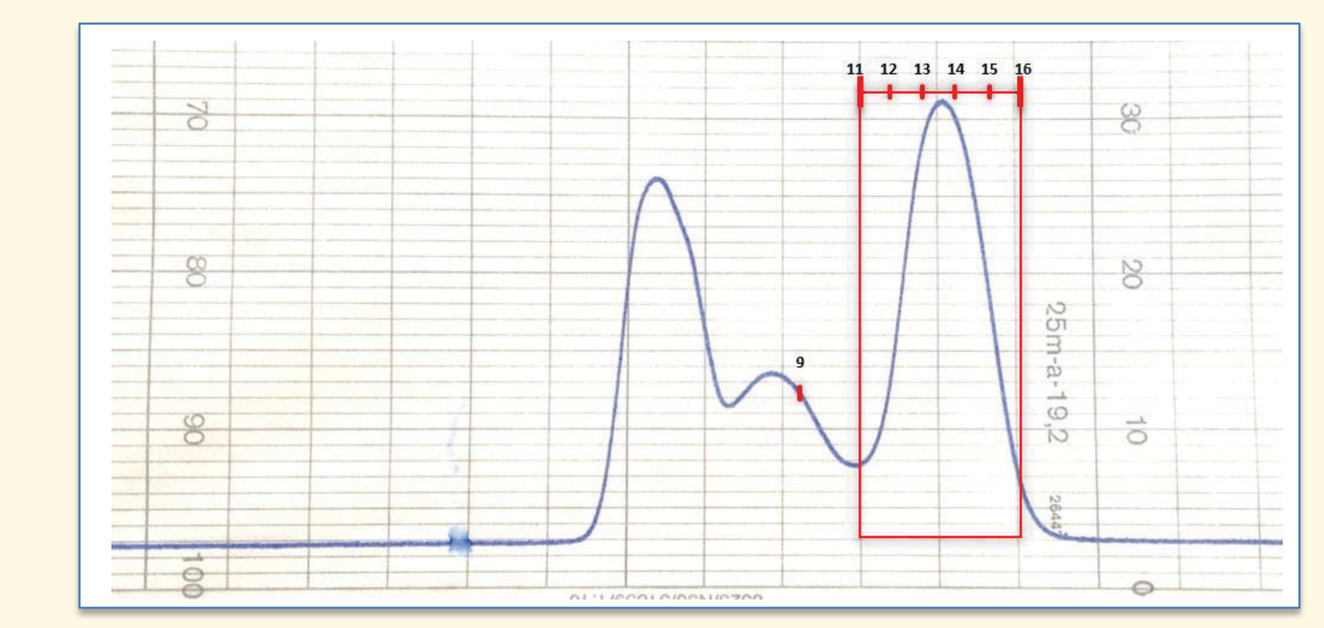


Figure 8 Klp61F–L5chimera Chromatograph. UV absorbance of 4mL fractions collected from the S100 gel filtration sizing column. The peak of interest (*red* box) corresponded to fractions 10-16.

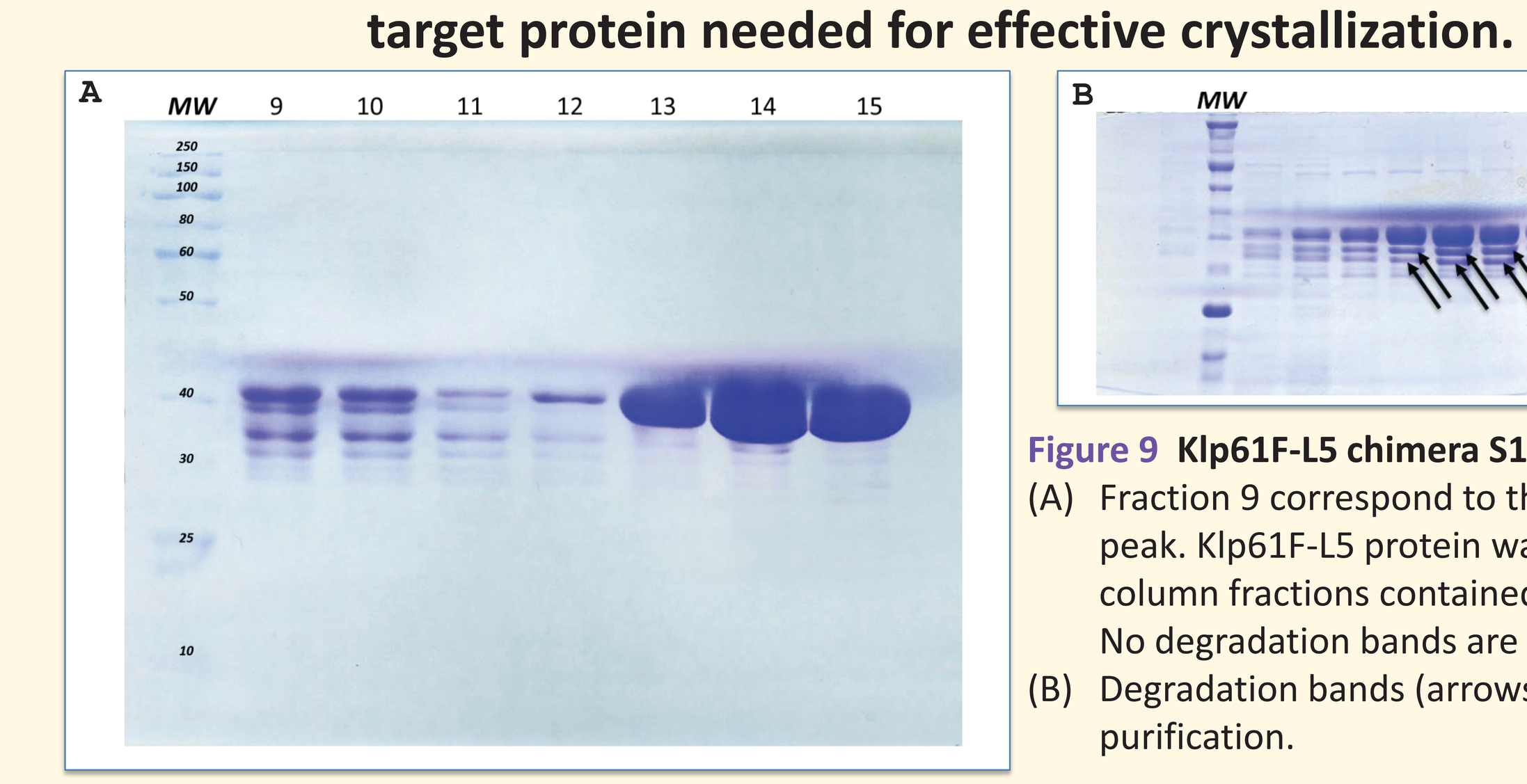
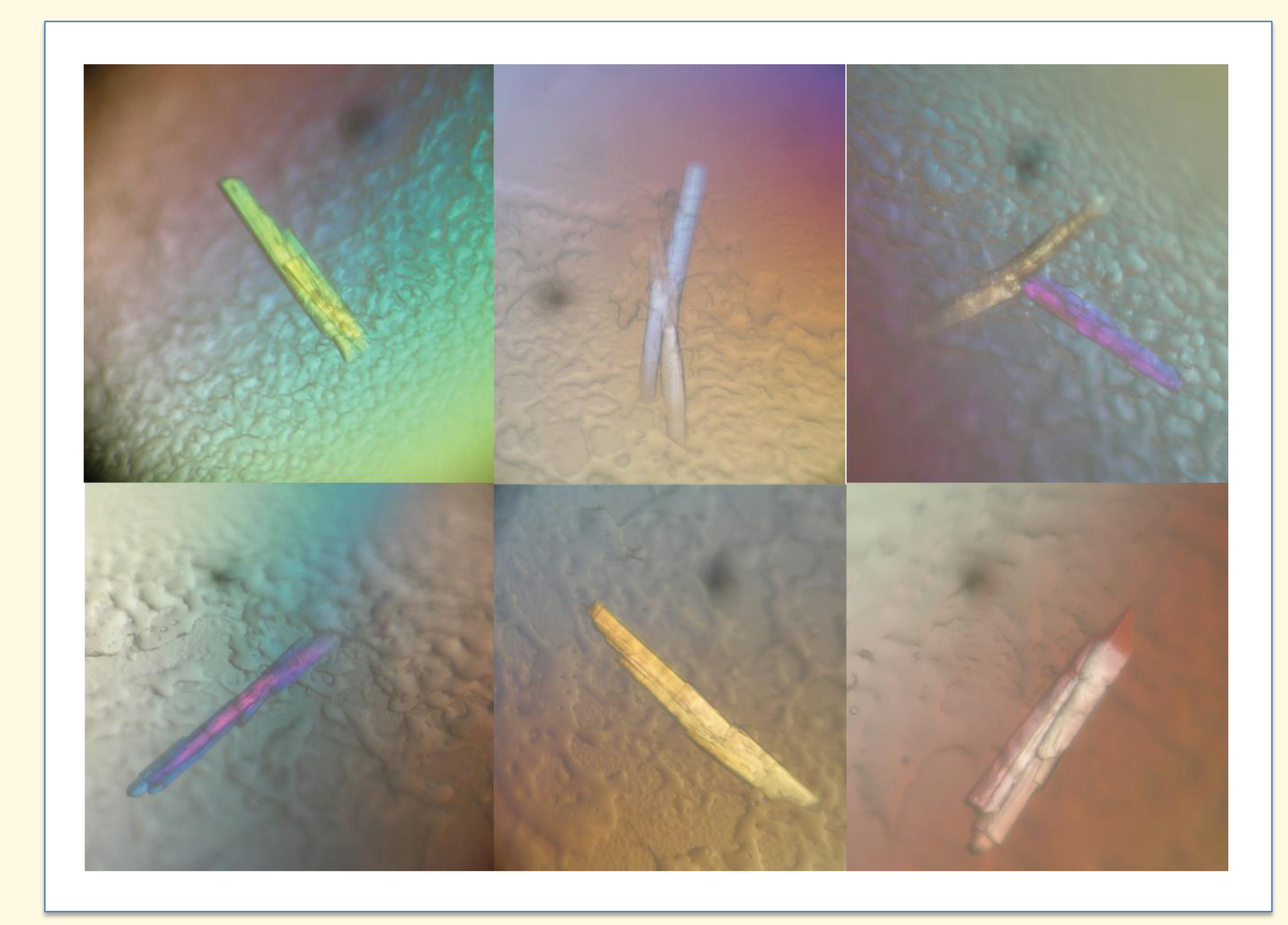


Figure 9 Klp61F-L5 chimera S100 sizing column fractions.

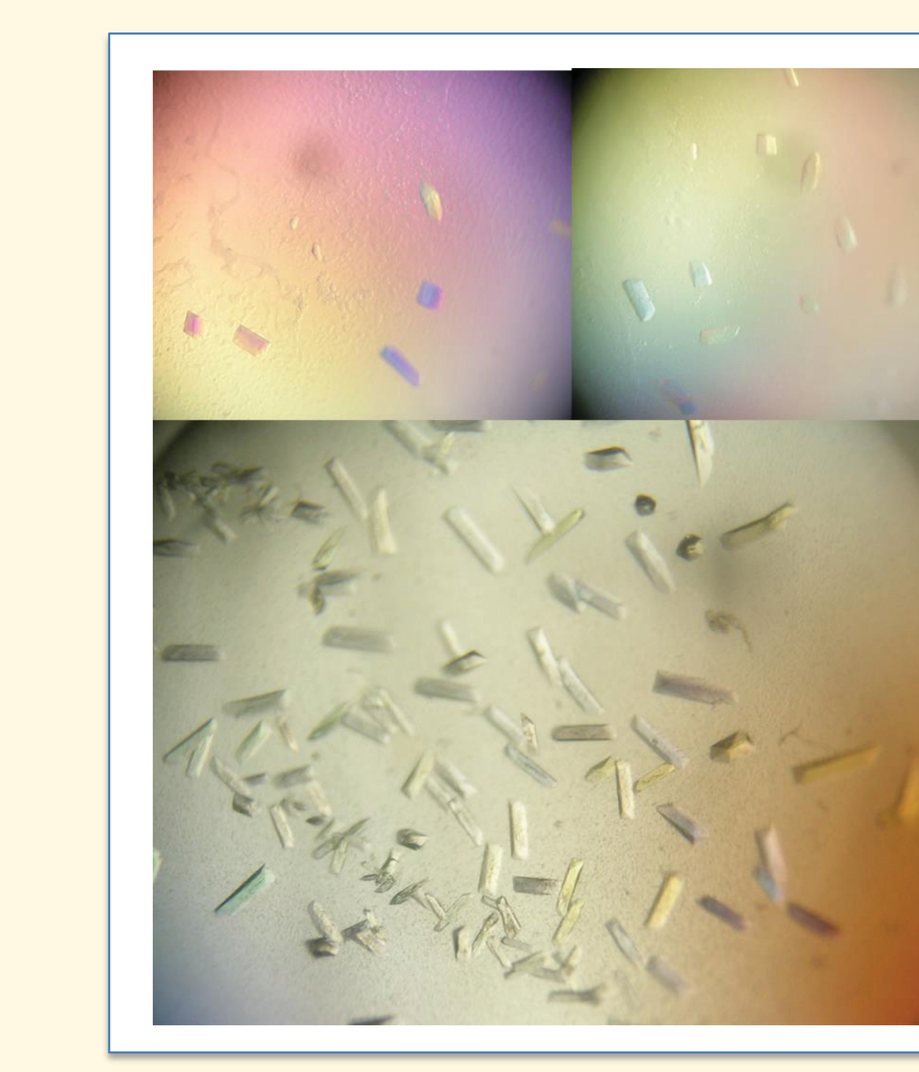
(A) Fraction 9 correspond to the earlier chromatograph peak. Klp61F-L5 protein was detected in the S100 column fractions contained within our peak of interest. No degradation bands are present.

(B) Degradation bands (arrows) seen in initial attempts at purification.



The Klp61F-L5 chimera protein crystals we obtained (*left*) need to be improved in order to yield a high-resolution structure of the Klp61F-L5 chimera motor domain-drug complex.

Despite using the same crystallization techniques and conditions, there is a noticeable difference from the Klp61F-L5 crystals obtained in previous successful crystallization experiments (*right*). We believe this could be due to a difference in purification protocol, or that we are using a different expression construct than that which was used in the prior effective crystallization trials.



Klp61F-L5 chimera protein crystals from effective crystallization trials. Our aim is to improve upon the initial diffraction data provided by these crystals.

Conclusions

- **Drug sensitivity can be engineered into a kinesin motor protein**
- **The mechanism of allosteric inhibition in human Eg5 is conserved in Klp61F, and may also be conserved in other kinesins.**
- **Allosteric inhibition may be reconstituted in all kinesins by targeting the corresponding L5 pocket or modeling the corresponding L5 loop after that of Eg5**
- **A high resolution structure of the Klp61F-L5 chimera motor domain-drug complex will reveal detailed information on the atomic-level contacts that mediate allosteric inhibition.**
- **Our results indicate that residue changes in the chimera L5 loop, although conservative, destabilize the chimera protein and alter proteolysis.**
- **Alterations in the protein purification process are needed to improve upon crystallization trials, which have the potential to inform our understanding of protein functions.**
- **Better understanding of kinesin protein function may lead to the development of molecular-targeted therapies for various human cancers.**