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Expression and Purification of Active Human Kinases Using *Pichia pastoris* as A General-Purpose Host

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Abstract

Background: The heterologous expression of human kinases in good purity and in a monomeric, soluble and active form can be challenging. Most of the reported successful attempts are carried out in insect cells as a host. The use of *E. coli* for expression is limited to a few kinases and usually is facilitated by large solubility tags that can limit biophysical studies and affect protein-protein interactions. In this report, we evaluate the methylotrophic yeast *Pichia pastoris (P. pastoris)* as a general-purpose host for expression of human kinases.

Methods: Six diverse kinases were chosen due to their therapeutic importance in human cancers. Tested proteins include serine/threonine kinases cyclin-dependent kinases 4 and 6 (CDK4 and 6) and aurora kinase A (AurKA), receptor tyrosine kinase erbB-2 (HER2), and dual specificity kinase mitogen-activated protein kinase kinase 3 (MKK3b). Noting that positively charged kinases expressed with higher yield, we sought to improve expression of two challenging targets, CDK6 and HER2, by fusing the highly basic, N-terminal domain of the secreted tyrosine-protein kinase VLK. The standard expression procedure for *P. pastoris was adopted*, followed by purification

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using affinity chromatography. Purity and activity of the proteins were confirmed and compared to published values.

Results: Some kinases were purified with good yield and purity and with comparable activity to commercially available versions. Addition of the VLK domain improved expression and decreased aggregation of CDK6 and HER2.

Keywords

Kinase; *P. pastoris* ; Yeast; Expression; Activity

Background

Protein kinases play a central role in cell regulation and signal transduction by selectively phosphorylating downstream effector proteins. Dysregulation of kinase activity as a result of mutations, altered expression levels or localization can lead to abnormal cellular behavior and cancer. Human kinases are important drug targets, comprising one of the largest groups of druggable targets in the human genome [1]. Structural studies on kinases have been essential for advancing drug design but have often focused on the catalytic domains; elucidation of the full-length kinases would allow structural studies of protein-protein interaction domains and other regulatory regions that affect catalytic activity and are often dysregulated in cancer [2, 3]. Although *E. coli* has been the major workhorse for recombinant protein expression, it has only limited success so far in producing human protein kinases, particularly full-length proteins [4]. The baculovirus expression system (BES) has become the system of choice for many kinases because it can consistently produce soluble and active enzymes in insect cells; however, the long turnaround time and high cost of insect cell expression systems are undesirable. Furthermore, due to its prohibitively high cost of isotope labeling, BES is not convenient for producing proteins for nuclear magnetic resonance (NMR). One notable example is the tyrosine kinase c-Abl, which saw a large increase in NMR studies after a bacterial expression method was developed [5]. The majority of kinases expressed with BES also use bulky protein fusions, most commonly maltose binding protein (MBP), thioredoxin (Trx), or glutathione-S-transferase (GST), which complicate the study of kinase protein-protein interactions and may impart non-native properties to the recombinant protein.

The methylotrophic yeast *Pichia pastoris (P. pastoris)* is a widely used host for recombinant protein expression, though it has not been routinely used for human kinases. *P. pastoris* is easy to manipulate genetically, with a strong promoter to drive expression of introduced genes. The target proteins are often expressed in high yield in cultures that only require simple conditions and affordable media for rapid growth [6]. As a eukaryote, *P. pastoris* is capable of correctly folding most eukaryotic proteins and producing them in a soluble and active form; yeast can incorporate post translational modifications, such as glycosylation, disulfide-bond formation and proteolytic processing. Relevant for production of kinases, this system has been successfully applied for the production of functional proteins that require phosphorylation for activity [7–9]. As a microorganism, *P. pastoris* is amenable to high-density fermentation and growth in a minimal medium, allowing easy scale up and isotope labeling. To date, the expression of kinases has been challenging in this system,

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perhaps because they phosphorylate intracellular proteins, leading to growth inhibition and toxicity to *P. pastoris*, among other hosts [10]. Active PINK1 has been expressed with a small purification tag [11]; however, other *P. pastoris*-expressed kinases utilized bulky tags [12] or failed to express in an active form [13]. To our knowledge, a general approach for expressing kinases in *P. pastoris* has not been reported.

Towards the goal of expressing minimally tagged, functional, full-length kinases (more specifically, their full-length cytoplasmic domains) at a reasonable cost, we developed an expression system in *P. pastoris*. The system has demonstrated potential as a general-purpose, protein-production platform using a variety of kinases, including cyclin-dependent kinases 4 and 6 (CDK4 and 6), aurora kinase A (AurKA), receptor tyrosine kinase erbB-2 (HER2), and dual specificity kinase mitogen-activated protein kinase kinase 3 (MKK3b). All six kinases were isolated in an active form, with varying levels of expression.

Results

Strategy for kinase expression in *P. pastoris*

We applied the same workflow for expression and purification for all target kinases. Constructs were prepared using the Gibson assembly method [14] and transformed into yeast for expression tests, as detailed in Methods. Each kinase was fused with the small purification tags Strep II (WSHPQFEK) or His10 separated from the protein sequence by a TEV cleavage site (Figure 1, Table 1). Individual transformants in *P. pastoris* typically produced different amounts of recombinant protein. We therefore screened multiple (4–10) single clones of the transformants using Western blotting, and the clone exhibiting the highest expression level was selected for scale-up. For co-transformation experiments, the two plasmids had the same selection marker (zeocin), thus colonies were screened using Western blotting to determine which colony had the highest expression for both kinases (Supplementary Figure 1).

Culture conditions were selected after testing several temperatures and times (Supplementary Figure 2) for their effect on protein yield. For the first 24 hrs of culture, yeast were grown at 30 °C in buffered glycerol-complex medium (BMGY) to accumulate biomass. After 24 hours, protein production was induced by adding 0.5% methanol and growing in buffered methanol-complex medium (BMMY) for 48 hrs at 28 °C. Cells were then harvested and stored at −40 °C or processed the same day as detailed in Methods. Proteins were then purified from crude lysate on affinity resin (Strep-Trap or HisTrap, depending on the tag used, see Methods); purity was evaluated by polyacrylamide gel electrophoresis (PAGE) and Western blotting was used to establish the identity of the protein band. Lysate of uninduced *P. pastoris* was included as a control (see Supplementary Figure 2 and 3). Purity was estimated from SDS-PAGE by densitometry, taking the ratio of the target protein band divided by the total protein. The yield of purified kinase was calculated based on the final amount of protein per liter culture (mg/L), reflecting the efficacy of the expression and purification process. Enzymatic activity was measured using the ADP-Glo Max *in vitro* kinase enzymatic assay kit (Promega), using the recommended substrates. Activity was compared to commercially available kinases with reported activity measured using the same substrates.

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Expression and purification of MKK3b

MKK3 (isoform b) is a cytosolic kinase widely involved in cellular signaling. This dual specificity kinase is part of the RAS/MAPK pathway, which plays a key role in regulating proliferation, differentiation and survival [15]. The active enzyme was expressed with a small Strep II tag in *P. pastoris* with good purity as judged by SDS-PAGE (Figure 2). After elution from a StrepTrap column, higher molecular weight aggregates were detected by SDS-PAGE. To purify the monomeric form, we utilized gel filtration chromatography as detailed in the Materials section. The fractions containing the monomeric soluble protein were collected and concentrated and the identity was confirmed by Western blotting with anti-Strep antibody (Figure 2C, D). The yield was 0.5 mg/L culture and the catalytic activity assessed by ADP-Glo Max was comparable to the activity reported for commercially available MKK3 using the same reported substrate (inactive p38) and assessed with radioactive assays (SignalChem, Table 1). The enzyme was previously expressed with a bulky MBP tag and His tag in *E. coli*; the inactive protein required co-expression with its upstream activator MEKK-C to obtain 25% yield of active enzyme [15,16]. Two other reports expressed a constitutively active mutant in BES with no reported yield [17,18].

Expression and purification of full-length AurKA

The mitotic kinase AurKA has gained attention as a target in several malignancies [19]. We therefore tested several systems to express recombinant, full-length AurKA. Full-length, His6-tagged AurKA did not express in a soluble form using the Rosetta strain of *E. Coli* (data not shown). The same construct suffered from degradation after attempted expression in SF9 insect cells (Supplementary Figure 3). Moving to the yeast *Saccharomyces cerevisiae* yielded soluble protein without degradation, but the yield was low (0.14 mg/L).

We successfully expressed and purified strep-tagged, full-length AurKA in *P. pastoris* using a single purification step (StrepTrap). In contrast to the other recombinant systems tested, expression in *P. pastoris* maintained solubility and purity, which improved the yield of purified protein by almost 7-fold (1 mg/L culture). The purified protein was a monomer, 95% pure on SDS-PAGE and active and the identity was confirmed by Western blotting with anti-AurKA antibody (Table 1, Figure 3). The enzymatic activity of the recombinant AurKA (using ADP-Glo Max) was approximately 25–30% of the commercially available kinase using the same substrate (MBP) and assessed with radioactive assays (18.7 nmol/mg/min vs 62–73 nmol/mg/min; SignalChem).

Expression and purification of CyclinD2 (CycD2), CDK 4 and 6

Cyclin-dependent kinases are involved in cell cycle regulation and are considered important targets in human cancers. The kinases are almost always co-expressed using BES with a cyclin to improve the kinase structural stability, folding and activity [20].

N-terminal Strep-tagged CDK4 and CDK6 were expressed in *P. pastoris* after co-transformation with N-terminal His6-CycD2 to obtain cyclin-CDK complexes. Complex formation was confirmed first with CDK4/CycD2 in a small-scale culture (100 ml) by dual-tag tandem affinity chromatography; the complex co-eluted from both StrepTrap and HisTrap columns as confirmed by SDS-PAGE and Western blotting with specific

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antibodies (Figure 4). Large scale (1 L) culture for protein expression of both complexes was processed on a StrepTrap column with higher purity and yield for the CDK6-CycD2 complex than the CDK4-CycD2 complex (0.30 mg/L, 95% purity vs 0.14 mg/L, 50% purity, respectively; Figure 5, Table 1). Further purification by HisTrap for CDK4-CycD2 complex did not improve the purity. Both CDK4 and 6 showed slightly improved activity compared to the commercially available versions, which were co-expressed with cyclin D1 in SF9 insect cells, using the Rb protein (residues 773–928) as substrate. Activity was 14.4 nmol/mg/min (ADP-Glo Max) vs 12 nmol/mg/min (radioactivity assay; SignalChem) for CDK4/cyclin and 17.1 nmol/mg/min (ADP-Glo Max) vs 8–9.3 nmol/mg/min (radioactivity assay; SignalChem) for CDK6/cyclin.

Attempted expression of the single proteins CDK4, CDK6 and CycD2 in *P. pastoris* with several tags was not as successful. Expression of CDK6 was not detected using the experimental protocol, despite several rounds of transformation and expression tests. CycD2 was expressed but was insoluble, while CDK4 expressed in a soluble form but with very low purity and yield (Supplementary Table 1). These results concur with published reports describing the difficulties in expressing the individual proteins [20].

We noted that the poorly expressing CDK4/6 and CycD2 were acidic proteins, with pI values of 6.08, 5.74, and 5.25, respectively (Table 1), while MKK3 was less acidic (pI = 6.28), and full-length AurKA was basic (pI = 9.25). The N-terminal domain of AurKA contributed to the overall basicity of the protein, and we hypothesized that addition of a basic domain would improve expression levels. To test this idea, we added a highly basic 110 amino acid N-terminal domain from the secreted kinase VLK (Molecular weight = 11.4 KDa, pI = 11.7) to the kinase domains of CDK6 and HER2 (see below) and monitored the effect on expression. In contrast to CDK6-Strep, which did not show expression in *P. pastoris* using our experimental protocol in the absence of CycD2, His10-VLK-CDK6-Strep did express (Supplementary Figure 1); however, subsequent purification on a StrepTrap column produced a protein with very low purity and was further purified after cleaving the His10-VLK tag as detailed in the Methods section (Figure 6). The identity was confirmed using Western blotting with specific antibody and the obtained protein was soluble and reasonably pure, albeit with low yield (0.05 mg/L, 70% Table 1). Given the difficulty of expressing CDKs without their cyclin protein partner, this result gave an initial indication that adding VLK fusion protein to shift the kinase pI could improve expression.

Expression and purification of HER2 kinase domain

HER2 receptor tyrosine kinase is a well-known oncogene whose cancer signaling is modulated by the catalytically inactive (pseudokinase) receptor HER3 [21, 22]. We designed several constructs encompassing the HER2 kinase domain with different sequence lengths and purification tags, with and without a point mutation V956R located in the its C-terminal lobe (Table 1, Supplementary Table 1). V956R blocks homodimerization of HER2, thus decreasing its activity and toxicity, consequently improving expression [23]. The kinase domain of HER2 (residues 705–1029) expressed in SF9 insect cells with a yield of 0.15 mg/L culture; however, longer constructs including part of the juxtamembrane segment located N-terminally to the kinase domain (JMB) and the C-terminal tail (residues 691–

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1255) did not express in SF9 (unpublished results). We therefore compared expression for short and long constructs in *P. pastoris* to determine whether yeast would allow expression of HER2 containing these protein-protein interaction domains.

Codon usage of the wild-type HER2 gene was optimized for *P. pastoris* without changing the amino acid composition (GenScript). HER2 kinase domain with the JMB and full-length C-terminal tail (residues 691–1255), with or without the V956R mutation and VLK-N-terminal sequence, was then expressed in *P. pastoris*. With a one-step purification on Strep-Trap columns, wildtype HER2 was purified to a modest but reproducible yield and purity in two purification trials (0.2 mg/L culture, 70%), with some aggregation (Figure 7). Adding the VLK fusion protein eliminated the aggregation but the protein was isolated with a lower yield and purity (0.05 mg/L culture, 40%). The V956R mutant of HER2 was purified with an apparent yield comparable to wild type but with lower purity (40%), as manifested by the higher molecular weight aggregates that could not be eliminated in subsequent chromatography steps. The elution of the wild-type construct on a size exclusion column was consistent with its being a monomer, and its identity was confirmed using SDS-PAGE and Western blotting with anti-HER2 antibody. Enzymatic activities (using ADP-Glo Max) of both the wild type and mutant HER2 were comparable to their commercial counterparts (using radioactive assay; Table 1).

Expression of the shorter HER2 kinase construct lacking the C-terminal tail (residues 705–1029) proved more challenging. While SF9 insect cells have produced high purity, active, protein in a monomer form with acceptable yield (unpublished results), expression and purification of this construct in *P. pastoris* suffered from aggregation, low yield and purity (Supplementary Table 1 and Supplementary Figure 4). Hence, SF9 and *P. pastoris* gave opposite results, with the long construct encompassing almost the entire intracellular portion of the receptor behaving better in yeast cells and the kinase-domain only constructs expressing better in insect cells.

Discussion

In the past two decades, several kinases have been targeted for anticancer therapy by inhibitors developed to bind their kinase active sites. More recently, drug discovery has also focused on inhibiting the protein-protein interactions that regulate homo/heterodimerization of kinases and kinase/substrate interactions [24–26]. These interactions can occur outside the kinase domain, increasing the need for studies of kinase constructs that include these regulatory domains. Recombinant expression and purification of such longer constructs has often been challenging, however.

We evaluated the yeast *P. pastoris* as an expression system for the production of active human kinases with short affinity tags. Emphasis was placed on expression of kinases including regulatory domains and kinases that have been difficult to express in *E. coli* or insect cells. *P. pastoris* combined the advantages of rapid growth and low cost with the ability to express eukaryotic proteins with proper protein processing and folding. Based on our reported activity of the expressed kinases, *P. pastoris* successfully phosphorylated the

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proteins, resulting in full or partial activation without the addition of external mammalian lysates or specific kinase activators.

For fifteen variations of six proteins, we observed a range of protein yield and purity (Table 1, Supplementary Table 1). The purified kinases were obtained in a monomeric, soluble and active form, usually after one-step affinity purification, which may be important for limiting aggregation. The yields obtained for even the most challenging targets were sufficient for enzymatic characterization. All targeted kinases had activity comparable to commercially available sources except for AurkA (Table 1); in this case the 2–3-fold lower activity level could be due to the absence of full phosphorylation-induced activation or due to the absence of activating proteins such as Tpx2, which may have been included in the commercially produced AurKA [27].

We observed that kinases with basic or slightly acidic pI (AurKA, MKK3) tended to have higher purification yields and suffered less aggregation than acidic proteins (HER2, CKD4). AurKA, in particular, represented a case study for the success of *P. pastoris* as an expression system. Previous AurKA reports expressed the kinase domain in *E. coli* and full-length AurKA in SF9, but the yields were not provided [19, 24, 28, 29]. Typically, AurKA has been recombinantly expressed as the catalytic domain, but we were interested in investigating the potential functions of the disordered N-terminal domain; thus, we opted to express a full length AurKA construct. While we could not directly compare the yield of AurKA purified from yeast and BES, the *P. pastoris* system was a more effective host to express the full-length kinase (Figure 3, Supplementary Figure 3).

Whether *P. pastoris* was the optimal expression host for MKK3 was unclear. While bacterial expression faced limited success, MKK3 has been expressed as a constitutively active mutant in insect cells at a yield of 8.6 mg/L culture [15–18]. The purification yield of wild type MKK3 in *P. pastoris* was lower (0.5 mg/L culture); however, we did not attempt to express the constitutively active mutant. Hence, when expressing a single kinase, it would be worthwhile to test multiple expression systems, as some kinases might be better expressed in *E. coli* or insect cells.

We succeeded in expressing kinases with low pI by modifying their expression constructs. For example, most of the intracellular domain of HER2 was obtained as an active and soluble monomer in moderate yields (0.2 mg/L, 70% purity) in *P. pastoris* but we did not detect expression in SF9 insect cells (unpublished results). By contrast, the isolated kinase domain suffered from aggregation and low purification yields in *P. pastoris* but expressed and purified successfully in SF9 cells (0.15 mg/L culture). For the most challenging targets, such as CDK6, we hypothesized that expression would improve if the pI were shifted from acidic to basic by addition of a cleavable N-terminal domain of VLK. Indeed, VLK fusion did improve expression of CDK6; although yield was low (0.05 mg/L culture, 70% purity), this approach offered the opportunity to obtain isolated CDK6 for protein-protein interaction studies.

Our expression system utilizes batch fermentation of *P. pastoris* grown in shake flasks by methanol induced expression [30]; however, *P. pastoris* can also be grown in continuous-feed

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fermentation bioreactors, which may provide an increase in cell mass and commensurate increase in protein yield, depending on the expressed protein and the bioprocess employed [31].

Conclusions

P. pastoris is a promising host for expression of soluble and active human kinases. The small, cleavable purification tags and simple purification procedures used here provide an approach to express active kinases that include domains outside the kinase domain, facilitating biophysical studies of protein-protein interactions and crystallization.

Methods

Chemicals and reagents

The vector pPICZ-A and Top10F' bacterial competent cells were from Invitrogen and specific antibodies were from Millipore and Cell Signaling. SF9 insect cells and ESF 921 media were from Expression systems. Strep-Trap, HisTrap and gel filtration columns (Superdex 16/600 200 pg) were from GE. All other chemicals unless otherwise stated were from Sigma Aldrich.

Design and construction of protein expression vectors

HER2 has shown low purification yields in insect cells as noted in Results. To alleviate possible causes of weak expression in *P. pastoris*, the genes of HER2 (residues 691–1255) and hVLK were codon-optimized for *P. pastoris* and ordered from Genscript Inc. The genes encoding the full-length AurKA, CDK4, CDK6, MKK3 and G1/S-specific cyclin (CycD2) were described previously [32]. Genes are positioned downstream of the methanol-inducible alcohol oxidase promoter of the pPICZ-A vector backbone that carried a zeocin selection marker. Affinity tags were added to all genes to facilitate purification (Table 1, Figure 1). For CDK6 and HER2, expression trials included a fusion construct with the N terminal hVLK (Figure 1). The prepared recombinant reactions were used to transform Top10F' competent cells, and plasmid DNA was prepared using Zyppy™ Plasmid Midiprep Kit (Zymo Research) following manufacturer's protocol and sent for sequencing (Elim Biopharm) to confirm the constructs.

P. pastoris host strain for expression

The host for protein expression is an in-house strain derived from the protease-deficient strain SMD1163 (his4, pep4, prb1) (Invitrogen) and denoted SMD1163H (pep4, prb1; Mut^{+}). The strain is obtained by transforming the SMD1163 host with the pPIC3.5K plasmid (AddGene plasmid #18707), which was linearized with SacI overnight. Transformation was performed as detailed in the "Transformation of *P. pastoris* expression host" section, below. pPIC3.5K was added to improve growth rates and to enable cells to grow in absence of histidine in the growth media.

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Transformation of *P. pastoris* expression hosts

P. pastoris electroporation-competent cells were prepared with a high-efficiency transformation protocol [33]. The transformation of host cells was carried out using the pPICZ-A plasmids containing the kinase constructs linearized with PmeI to facilitate transformation. Twenty to thirty μg of linearized plasmid was transferred to a pre-chilled 0.2 cm electroporation cuvette, mixed with 40 μl competent cells, and incubated on ice for 5 minutes. Electroporation was conducted on a Bio-Rad Gene Pulser II electroporator with pre-set parameters for *P. pastoris* (2000V, 200Ω, 25μF). After electroporation, cells were resuspended in a mixture of 1 ml of 1 M sorbitol and yeast extract peptone dextrose (YPD) and were allowed to recover at 30°C for 1.5 hours. Two hundred μl of the mixture was plated on a YPD-Sorbitol (YPDS) plate with 0.5 mg/ml Zeocin. The plates were incubated at 30°C without light for 3 days, until colonies appeared.

To select for multi-copy inserts, we employed the post-transformation vector amplification (PTVA) method [34]. Briefly, all colonies from the YPDS plate containing 0.5 mg/ml zeocin were scraped, pooled in 1 ml sterile water, and further diluted into 10 ml sterile water. One hundred and fifty μl diluted suspension was plated on a fresh YPDS plate with 1 mg/ml zeocin. The plate was incubated without light for another 3 days until colonies appeared. If more than 100 colonies were obtained, the process was repeated with 2 mg/ml zeocin until the number of colonies was between 10 and 20.

Antibodies and Western blotting

For electrophoretic gel separation, samples were collected and diluted as indicated in each figure, Protein samples were then denatured by boiling in 6X Laemmli sample buffer and run on 4–12% gradient denaturing polyacrylamide gel. For Western analysis, preassembled iBlot® Gel Transfer Stacks were used to transfer proteins from gels to PVDF membranes using the default P3 preprogrammed run (20 V for 7 minutes). Western blots were carried out on the iBind™ Flex system (Invitrogen) per the manufacturer's protocols. Briefly, membranes were equilibrated with 1X iBind™ Flex Solution, Flex cards were wetted and the primary and secondary antibodies diluted per manufactures' recommendations. The antibodies and wash solutions were placed in the appropriate wells and the immunostaining proceeded for 2.5 hours as recommended by the manufacturer. The target proteins were probed with anti-kinase antibodies and/or anti-tag antibodies as indicated in each figure. The antibodies were purchased from Cell Signaling (CDK4 (DCS156) #2906, CDK6 (DCS83) #3136) Santa Cruz Biotechnology (CycD2 (B-6) #sc-376676), Millipore Sigma (Strep-Tag II HRP Conjugate #71591–3, HER2 (24B5) #04–291, AurKA (35C1) # A1231). Primary untagged antibodies were further probed with a secondary anti-mouse HRP-linked Antibody (Cell signaling #7076). Chemiluminescence detection was done using Thermo Scientific™ Pierce™ ECL Western Blotting Substrate per the manufacturer's protocol.

Expression of kinase targets in *P. pastoris*

Positive transformants were picked and colony PCR was performed using standard AOX sequencing primers (Forward: GACTGGTTCCAATTGACAAGC, Reverse: GCAAATGGCATTCTGACATCC). After confirmation of gene integration into the yeast genome, single transformed colonies were screened for target protein expression by

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inoculation in 5 ml BMGY and overnight growth. Cells were then pelleted, washed, and resuspended in 5 ml BMMY with 0.5% methanol and grown for 48 hours at 28°C for protein production; 0.5% methanol was replenished after 24 hours. Aliquots were collected at 24 and 48 hrs to measure biomass and protein production levels in order to assess toxicity and optimal incubation time for protein production. Proteins were then isolated by alkaline extraction by incubating cells for 10 minutes in 0.1 M NaOH, pelleting and resuspending in Laemmli sample buffer after normalization to 1 OD and then separated by gel electrophoresis. Samples were electrotransfered to PVDF membrane using an iBlot™ system and analyzed by Western blot (iBind™ system, Invitrogen) per the manufacturer's protocols (see above). The transformant with the highest expression level was chosen for large-scale 1 L culture protein production.

Protein production and purification

All kinases reported in this study were expressed on a 1-liter scale under the same conditions. A single colony from the highest expressing transformant of each target kinase was used to inoculate 20 ml of BMGY medium. This culture was incubated with vigorous shaking at 30°C for 16–18 hours. Cells were pelleted, washed, and resuspended in 1L BMMY medium and grown for 48 hours at 28°C for protein production. Cells were then harvested, resuspended in Buffer A (100 mM Tris HCl pH8, 150 mM NaCl, 10% glycerol, 1 mM TCEP, 0.01% Tween) freshly supplemented with 2 mM PMSF and protease inhibitor cocktail tablet and lysed in a Bead-beater (SPEC) following the manufacturer's instructions. The cell lysate was clarified by centrifuging at 21000 rpm for 1 hour. The supernatant was filtered through a 2-μm prefilter and a 0.45-μm filter then loaded onto an ÄKTA FPLC system with 3×5 ml StrepTrap columns that had been equilibrated with Buffer A. After loading, the columns were further washed with 3 column volumes of Buffer A. The protein was eluted with 3 column volumes of Buffer B (Buffer A supplemented with 5 mM D-desthiobiotin). The eluent was pooled, concentrated and buffer exchanged (Amicon Ultra Centrifugal Filters) into 20 mM Tris HCl pH 8, 75 mM NaCl, 2% glycerol and 1 mM TCEP. The concentration, purity and identity of target proteins in the eluted fractions were estimated by measuring OD_{280}, SDS-PAGE with Coomassie staining, and Western blots, respectively.

MKK3 purified from the StrepTrap column was subjected to a second purification step on gel filtration to separate the monomeric protein form. The eluent from the first purification was pooled, diluted in buffer (50 mM Tris HCl pH8.5, 150 mM NaCl, 5% glycerol and 1 mM TCEP) and injected onto a gel filtration column (HiLoad 16/60 Superdex 200).

To remove the His10-VLK tag from CDK6, the eluent from StrepTrap column was pooled, diluted 10-fold in buffer (50 mM Tris HCl pH8, 300 mM NaCl, 10% glycerol and 1 mM TCEP) and injected to an equilibrated 5 ml HisTrap column. After washing three times with increasing imidazole concentration (20 – 80 mM), the protein was eluted using 250 mM imidazole with improved purity. The eluent was pooled and incubated overnight with in-house purified TEV protease to cleave the His10-VLK portion, then passaged over a 1 ml His resin gravity column to elute CDK6-Strep in the flow through.

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Expression in SF9 insect cells

Expression in SF9 cells was done using Bac-to-Bac expression system (Gibco BRL) as detailed in the manufacture's protocol and in previous publications [21, 35]. Cells were harvested and lysed using a microfluidizer in lysis buffer (25 mM Tris (pH 8.0), 10 mM NaCl, 5% glycerol, 10 mM imidazole, 3 mM β-mercaptoethanol) freshly supplemented with protease inhibitor cocktail tablet. Five ml HisTrap column equilibrated with the same buffer was used for the purification and elution was done using an imidazole gradient (20–250 mM).

Protein activity assay

Kinase specific activity was measured using the ADP-Glo Max assay protocol. Briefly, 5 μl of the primary kinase reaction was prepared in a 384-well plate by adding 2 μl of a series of concentrations of the kinases (50 – 400 ng final protein) in 1X kinase buffer (Cell Signaling), 2 μl of 250 μM high purity ATP mixed with the appropriate substrate and 1 μl of 5X kinase buffer. The substrates were purchased from SignalChem and included: 0.5 μg/μl peptide substrate (AAEEIYAARRG) for HER2 constructs, 0.1 μg/μl Retinoblastoma protein (Rb) for CDK4-CycD2 and CDK6-CycD2 complexes, and 0.1 μg/μl Myelin Basic Protein (MBP) for AurKA. Activity of MKK3 was measured indirectly by using MKK3 to activate 0.2 μg/μl inactive p38γ (Thermo Scientific). Two μl of this reaction mixture was added to 1 μl of 5X kinase buffer and 2 μl of 250 μM high purity ATP, mixed with 0.1 μg/μl MBP. Reactions were allowed to proceed for 45 min at room temperature, then 5 μl ADP-Glo Reagent was added and incubated for 40 minutes to terminate the reaction and deplete the unconsumed ATP. Finally, 10 μl of ADP-Glo Max Detection Reagent was added and incubated for 60 min. The resulting luminescence was detected using a Tecan Infinite F200 PRO multimode plate reader. A calibration curve was constructed with high purity ATP/ADP mixtures to quantify the ADP produced in the kinase reaction. The specific activity of each kinase was calculated by dividing the number of moles of ADP produced by the reaction time in minutes multiplied by the enzyme amount in mg. The resulting activity was compared when possible to the reported activity of commercially available kinases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights:

- Using *Pichia pastoris* as an expression host for active human kinases
- Improve expression of some HER2 constructs compared insect cell expression
- Expression of active full-length Aurora kinase A
- Enable expression of active CDK6 by adding VLK-fragment

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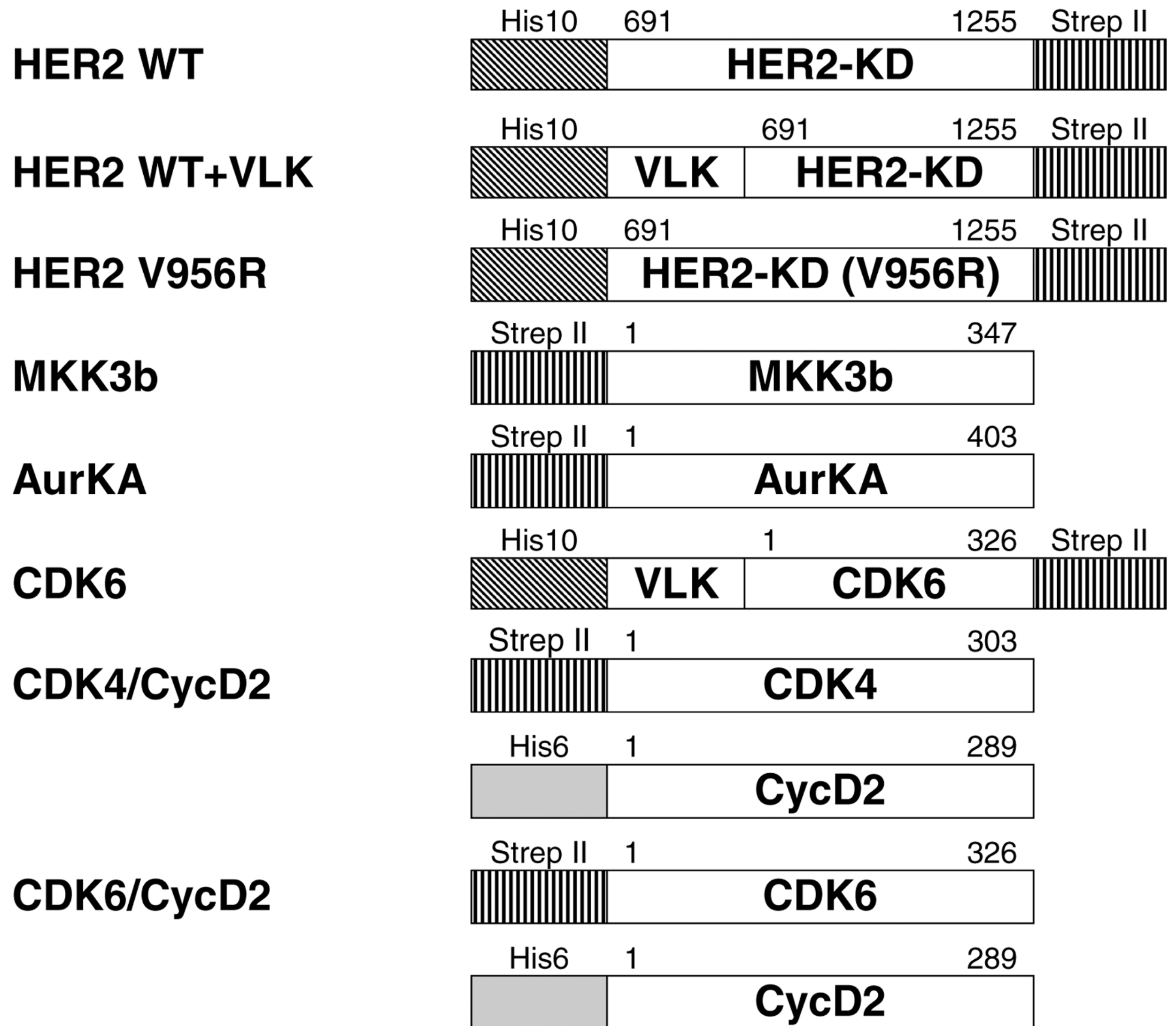

Figure 1:
Constructs of kinases expressed in *P. pastoris*

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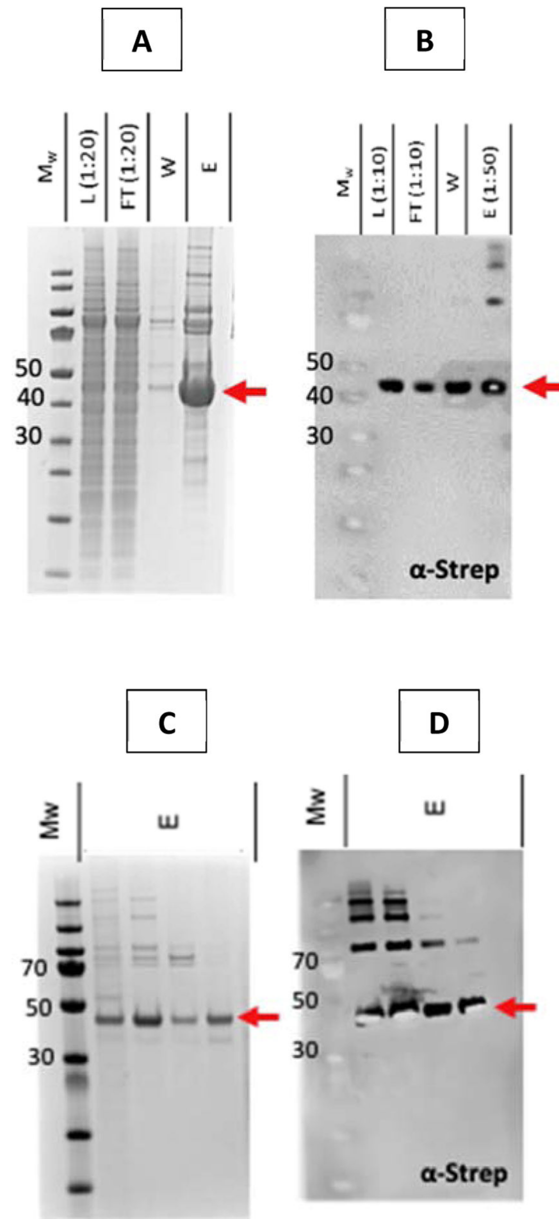

Figure 2:

Purification of MKK3b: A) Coomassie stain and B) Western blot for the purification. C) Coomassie stain and D) Western blot for gel filtration. M_w is the molecular weight marker, L is the clarified lysate, FT is the flow through, W is the wash and E is the column eluent, dilutions indicated to avoid overloading the gel (α- stands for anti-, determining the specificity of the used antibody).

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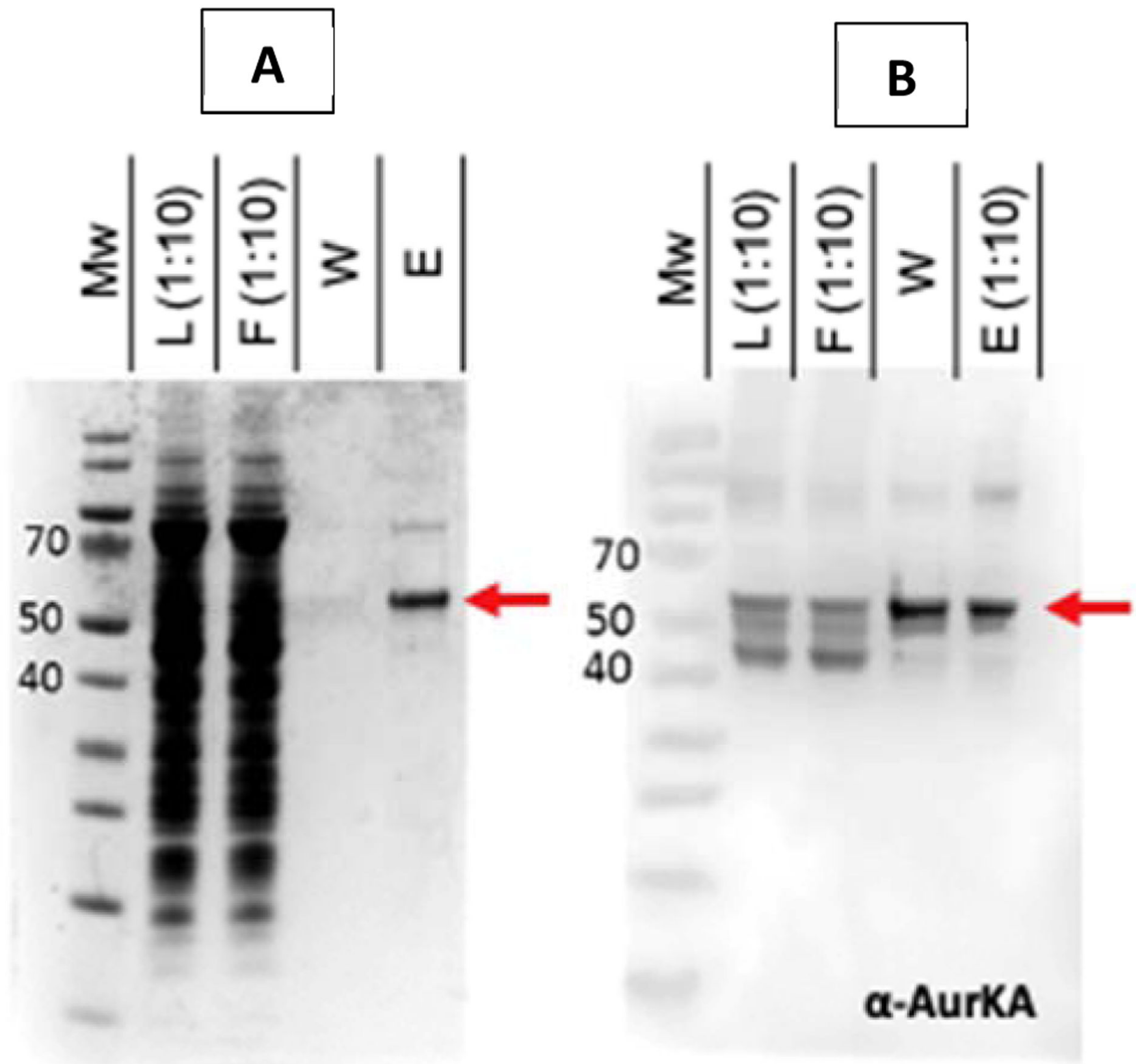

Figure 3:

Purification of AurKA: A) Coomassie stain and B) Western blot for the purification. M_w is the molecular weight marker, L is the clarified lysate, F is the flow through, W is the wash and E is the column eluent, dilutions indicated to avoid overloading the gel.

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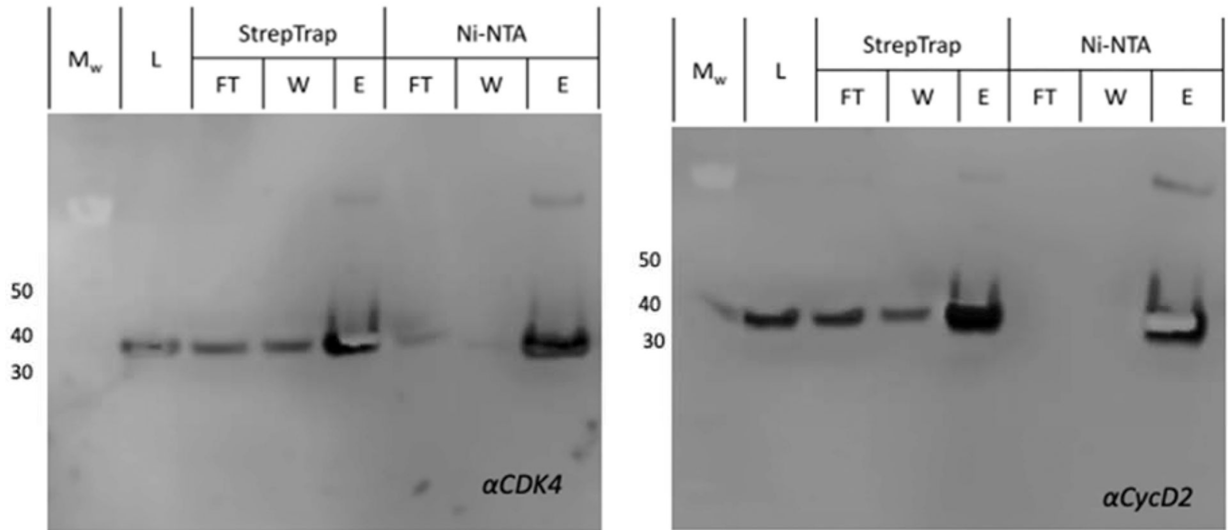

Figure 4:

Western blots with specific antibodies for the small-scale purification of the CDK4/CycD2 complex showing co-elution of the two proteins from tandem purification steps on Strep-Trap and Ni-NTA columns. M_w is the molecular weight marker, L is the clarified lysate, FT is the flow through, W is the wash and E is the column eluent.

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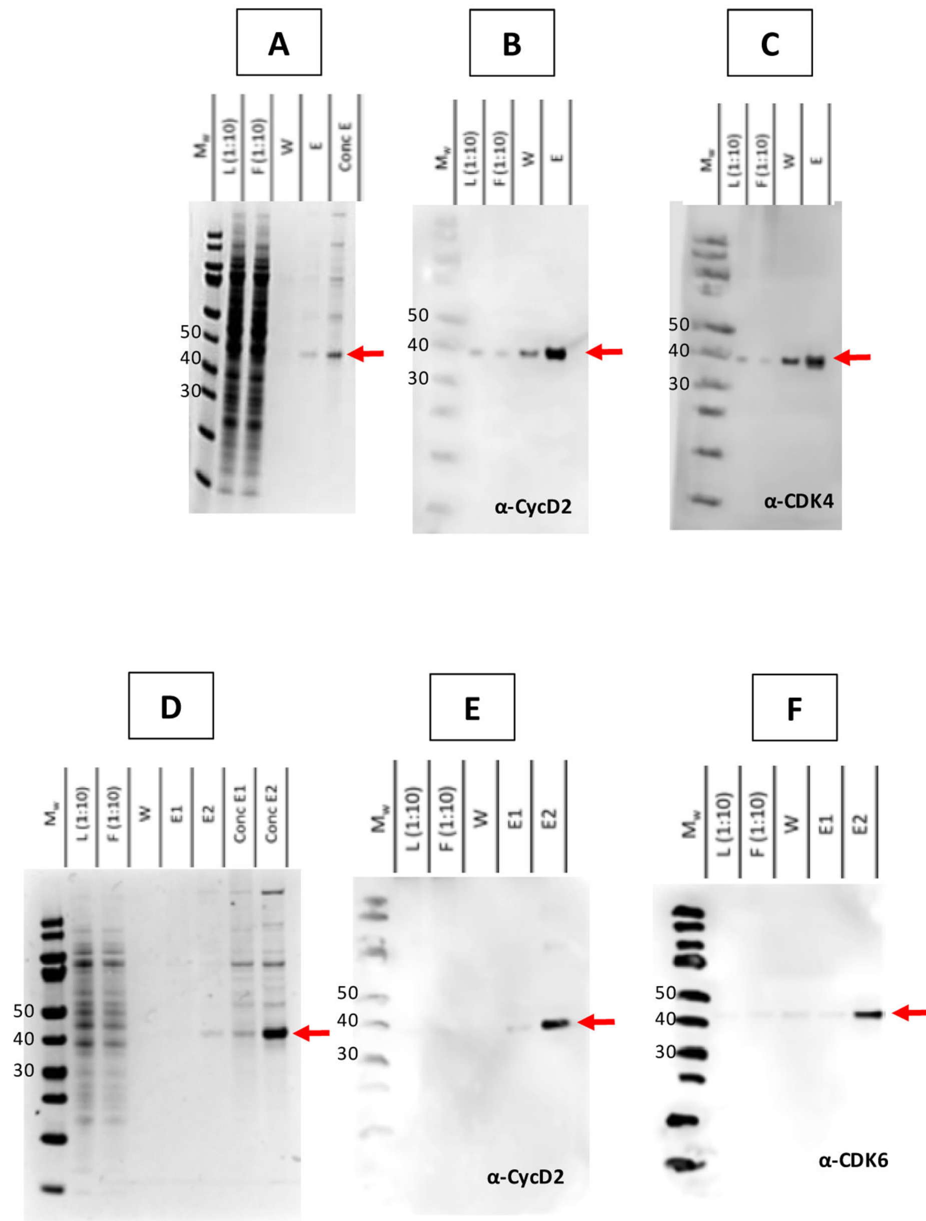

Figure 5:

Purification of CDKs/CycD2 complexes: A, B, C) show CDK4/CycD2 and D, E, F) show CDK6/CycD2. A and D) Coomassie stains and B, C, E and F) are Western blots for the complexes' purifications using specific indicated antibodies. M_w is the molecular weight marker, L is the clarified lysate, F is the flow through, W is the wash and E is the column eluent, dilutions indicated to avoid overloading the gel.

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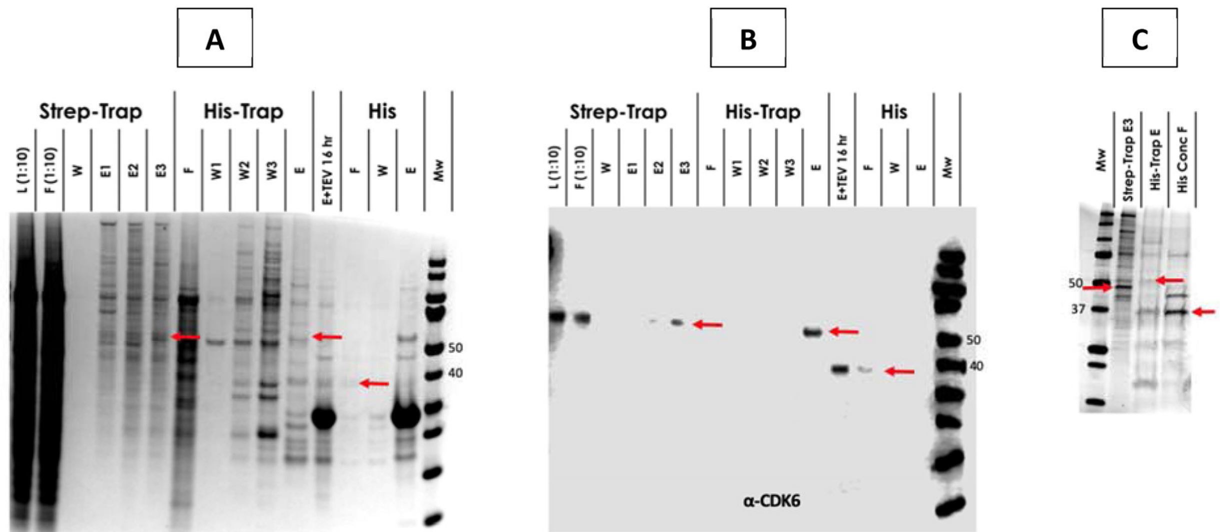

Figure 6:

Purification of VLK-CDK6. A) Coomassie stain for the three steps including His resin and B) Western blot for the same gel, C) concentrated elution from the three columns to show progression of purification. M_w is the molecular weight marker, L is the clarified lysate, F is the flow through, W1–3 are the progressive washs and E is the column eluent, dilutions indicated to avoid overloading the gel.

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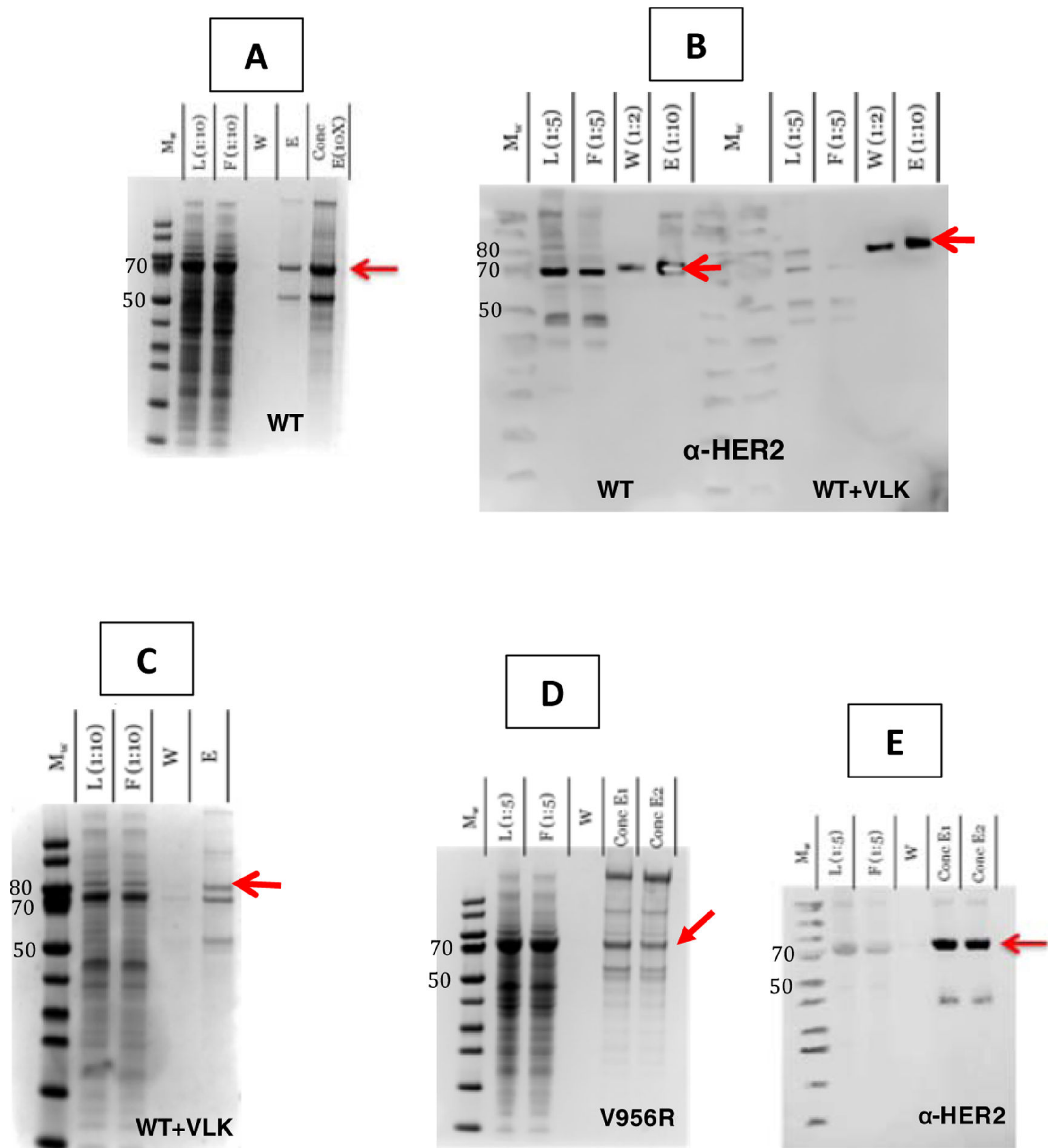

Figure 7:

Purification of HER2 constructs: (WT, WT+VLK and V956R) A, C, and D) Coomassie stains for indicated constructs, B) Western blot comparing the purification of the WT and WT+VLK, E) Western blot for the purification of V956R. M_w is the molecular weight marker, L is the clarified lysate, F is the flow through, W is the wash and E is the column eluent, dilutions indicated to avoid overloading the gel.

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Table 1:

Kinases Expressed in *P. pastoris*

Protein construct		Tags		Mwt (Kda)	pI	*P. pastoris* expression				Characterization data	
		N-term	C-term			Agg.	Yield %	Purity	Dispersity	Kinase specific activity (nmol/mg/min)	
										Measured	Reported[∞]
HER2 (691–1255)	WT	His10	Strep	66.8	5.24	Low	0.20 mg/L	70%	monomer	15.7	10–13
	WT with VLK	His10	Strep	77.4	5.88	N	0.05 mg/L	40%	monomer	ND	
	V956R	His10	Strep	66.9	5.28	Low	0.23 mg/L	40%	monomer	11.4	
MKK3b		Strep	-----	42.6	6.28	Low	0.50 mg/L	95%	monomer	32.6	24–42
AurKA		Strep	-----	49.1	9.25	Low	1.00 mg/L	95%	monomer	18.7	62–73
CDK6	with VLK	His10	Strep	52.3	8.57	Low	0.05 mg/L	70%	monomer	ND	
CDK4/CycD2		#	-----	37.0/36.1	6.08/5.25	N	0.14 mg/L	50%	Co-eluted	14.4	12
CDK6/CycD2		#	-----	40.2/36.1	5.74/5.25	N	0.30 mg/L	95%	Co-eluted	17.1	8–9.3

[∞] Commercially available sources (in text)

ND Not determined due to low yield and/or purity

[#] N terminal His6 on CycD2 and N-terminal Strep on both CDKs