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Tools and techniques

The *Aspergillus nidulans bimC4* mutation provides an excellent tool for identification of kinesin-14 inhibitors

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ABSTRACT

Centrosome amplification is a hallmark of many types of cancer cells, and clustering of multiple centrosomes is critical for cancer cell survival and proliferation. Human kinesin-14 HSET/KFIC1 is essential for centrosome clustering, and its inhibition leads to the specific killing of cancer cells with extra centrosomes. Since kinesin-14 motor domains are conserved evolutionarily, we conceived a strategy of obtaining kinesin-14 inhibitors using *Aspergillus nidulans*, based on the previous result that loss of the kinesin-14 KlpA rescues the non-viability of the *bimC*4 kinesin-5 mutant at 42 °C. However, it was unclear whether alteration of BimC or any other non-KlpA protein would be a major factor reversing the lethality of the *bimC*4 mutant. Here we performed a genome-wide screen for *bimC*4 suppressors and obtained fifteen suppressor strains. None of the suppressor mutations maps to *bimC*. The vast majority of them contain mutations in the *klpA* gene, most of which are missense mutations affecting the C-terminal motor domain. Our study confirms that the *bimC*4 mutant is suitable for a cell-based screen for chemical inhibitors of kinesin-14. Since the selection is based on enhanced growth rather than diminished growth, cytotoxic compounds can be excluded.

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1. Introduction

The cancer cell-specific centrosome clustering process has recently emerged as a target for potential anti-cancer drugs (Korzeniewski et al., 2013). Centrosome amplification, i.e., an abnormal increase in centrosome numbers, is a hallmark of many types of cancer cells, and it has also been associated with cancer aggressiveness (Brinkley, 2001; Marx, 2001; Pihan et al., 2001, 2003; D'Assoro et al., 2002a,b; Ogden et al., 2013; Godinho and Pellman, 2014; Godinho et al., 2014). Cancer cells with supernumerary centrosomes almost always undergo a process in which centrosomes are clustered into two poles before mitosis for bipolar cell division, which is critical for cancer cell proliferation yet causes chromosomal segregation errors that subsequently lead to more mutations (Gergely and Basto, 2008; Kwon et al., 2008; Ganem et al., 2009; Crasta et al., 2012; Zhang et al., 2015). A protein essential for centrosome clustering is HSET (also known as KIFC1), a minus-end-directed microtubule motor that belongs to the kinesin-14 family (Kwon et al., 2008). Importantly, as HSET is not essential for proliferation of normal cells with two centrosomes, HSET RNAi specifically kills cancer cells with extra centrosomes (Kwon et al., 2008). Small-molecule inhibitors of HSET/KIFC1 have been recently found using enzyme activity-based screens and iterative cycles of medicinal chemistry (Watts et al., 2013; Wu et al., 2013; Yang et al., 2014). However, their clinical efficacy has not been evaluated, and the possibility of off-target cytotoxicity has not been eliminated.

We have conceived a cell-based screening strategy using *Aspergillus nidulans* for obtaining non-cytotoxic kinesin-14 inhibitors. This is based on the notion that loss of kinesin-14 KlpA function in *A. nidulans* partially rescues the non-viability of the

Abbreviations: bim, blocked in mitosis; klp, kinesin-like protein; sbc, suppressor of *bimC*4.

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temperature-sensitive (ts) *bimC*4 mutant of kinesin-5 (O'Connell et al., 1993). As first revealed in *Saccharomyces cerevisiae*, the microtubule minus-end-directed kinesin-14 antagonizes the plus-end-directed kinesin-5 during mitotic spindle assembly (Saunders and Hoyt, 1992). While loss of kinesin-5 causes a collapse of the bipolar spindle, leading to monopolar spindle formation, mitotic failure and cell death, loss of kinesin-14 attenuates these defects (Saunders and Hoyt, 1992; O'Connell et al., 1993; Mountain et al., 1999; Wu et al., 2013). Functions of these mitotic kinesins are highly conserved evolutionarily from fungi to humans. In fact, the function of kinesin-5 in mitosis was first discovered in *A. nidulans* based on the genetic study on the *bimC*4 ts mutant (Enos and Morris, 1990). In *A. nidulans*, the kinesin-14 KlpA is not essential for mitosis and its loss partially suppresses the lethality of the *bimC*4 mutant at 42 °C (O'Connell et al., 1993). Thus, kinesin-14 inhibitors can be identified based on their ability to allow the *bimC*4 mutant to grow at 42 °C. Because the motor domains of kinesin-14s are highly conserved evolutionarily, we believe that a cell-based high-throughput screen using the *bimC*4 mutant will be an ideal way of obtaining kinesin-14 inhibitors. Since the selection will be made based on enhanced growth rather than diminished growth, cytotoxic compounds would be excluded.

In order to use the *bimC*4 mutant for kinesin-14 inhibitor screening, it is important to know if factors affecting BimC itself or other non-KlpA motor proteins could also reverse the growth defect of the *bimC*4 mutant. The nature of the *bimC*4 mutation is not known, however, because *bimC*4 is a ts mutant that grows normally at the permissive temperature of 32 °C, the mutant protein must be made and functional at 32 °C. Thus, it is possible that factors affecting the folding or the function of the BimC protein may result in reversion of *bimC*4 lethality at 42 °C, which would make the *bimC*4-based screen less effective. To address this possibility, we performed a genome-wide screen for genetic suppressors that allowed the *bimC*4 mutant to grow at 42 °C. Our result shows that no *bimC*4 intragenic suppressor was found, and importantly, the vast majority of suppressors contain KlpA kinesin-14 mutations affecting the conserved C-terminal motor domain. Our study further supports the idea that the *bimC*4 mutant is ideal for a high-throughput screen for inhibitors of kinesin-14.

2. Materials and methods

2.1. A. nidulans strains, media, mutagenesis and genetic crosses

We first crossed the LBA44 strain (*bimC*4; *alcA*(p)::GFP-*tubA*::*pyr*4; *pyroA*4; *pyrG*89; *wA*2) with XX222 (GFP-*nudA*^{HC}; *argB2::[argB***-alcAp*::mCherry-*RabA]; pantoB100; yA2*) (Abenza et al., 2009; Zhang et al., 2010) to generate the XX315 strain (*bimC*4; *alcA*(p)::GFP-*tubA*::*pyr*4; *argB2::[argB***-alcAp*::mCherry-*RabA]; pyroA*4, *yA*2; possibly GFP-*nudA*^{HC}). XX315 was used for mutagenesis, and thus, all the original *bimC*4 suppressor strains were all derived from XX315. UV mutagenesis on spores of *A. nidulans* strains was done as previously described (Willins et al., 1995; Xiang et al., 1999). The mutagenized spores were plated out on YAG (yeast extract + agar + glucose) rich medium and incubated at 42 °C for 3–4 days. For genetic analyses of the suppressor mutations, the original suppressors were crossed to a strain containing wild-type *bimC* (HookA-GFP-Af*pyrG*; *argB2::[argB***-alcAp*::mCherry-*RabA]; pabaA*1; possibly Δ*nkuA::argB;* possibly *pyrG89*) (Zhang et al., 2014). Sexual spores from the crosses were plated out on minimal medium and each progeny was replica plated at 32 °C and 42 °C on YAG medium. The appearance of the non-viable *bimC*4-like progeny at 42 °C indicates that the suppressor mutation is not linked to the *bimC* gene. To determine if the suppressor mutations were genetically linked to *klpA*, we crossed the original suppressor (*sbc*#/*bimC*4) strains with the MO61 strain containing the Δ*klpA* allele (Δ*klpA-pyr*4 or *klpA*1; *argB*2; *nicA*2; *pabaA*1) (O'Connell et al., 1993). Sexual spores from the crosses were plated out on YAG + Arginine medium and colonies at 32 °C were point-inoculated on the same medium to be incubated at 42 °C. The appearance of any non-viable *bimC*4-like progeny at 42 °C would suggest that the suppressor mutation is not in the *klpA* gene.

2.2. Genomic DNA preparation, PCR and sequencing analysis

Genomic DNA was prepared using the Dneasy Plant Mini Kit from Qiagen, Inc. (Valencia, CA, USA). The AccuPrime™ *Taq* DNA Polymerase from Invitrogen™-Life Technologies, Inc. (Grand Island, NY, USA) was used for polymerase chain reactions (PCRs) to generate the ~3 kb genomic DNA template from each suppressor strain. The set of primers used for PCR were KLPAF1 (ACCTCACATCATTCGCATAC) + KLPAR1 (GTGACTGGAGTCTAAACATC AC), or KLPAF0 (AAAGATAGCTCCCCCACTC) + KLPAR0 (GCAACAT CTCCAAAAGACAG). For sequencing, we used these primers plus two other primers, KLPAF2 (CGTTCCCTGGTGAAATTAT) and KLPAF3 (GGAACGAAAGAACACCAATA). Sequencing was done using the DNA sequencing service of Quintara Biosciences (Allston, MA, USA). Analyses on the sequencing results were done using MacVector 11.0.4 (MacVector, Inc. Cary, NC, USA).

3. Results

3.1. Fifteen bimC4 suppressors were obtained

To obtain *bimC*4 suppressor mutations, we first collected asexual spores of the *bimC*4 mutant grown at the permissive temperature of 32 °C. We performed UV mutagenesis on the spores and spread them on solid medium at the restrictive temperature of 42 °C. After 3–4 days, we selected colonies and inoculated them on new plates that were incubated at 42 °C for 3 days. None of the colonies formed at 42 °C had asexual spores, suggesting that the suppression is partial, which is similar to that caused by deletion of *klpA*. The new colonies were selected and inoculated at the permissive temperature of 32 °C to allow formation of healthy colonies with asexual spores. To confirm the suppressor phenotype, we inoculated the spores onto plates and incubated them at 42 °C for one more round of testing. Only those strains that formed colonies again at 42 °C were kept for further analyses. During these experiments, we also obtained several spontaneous suppressors that formed colonies at 42 °C without going through UV mutagenesis. These were tested for stability the same way as for the UV-generated suppressors, and then the two classes were combined for further analyses. At this stage, we collected a total of 15 suppressor strains, and they are named as *sbc*1/*bimC*4, *sbc*2/*bimC*4, *etc*. (*sbc* stands for suppressor of *bimC*4). The colony phenotypes of the strains are shown in Fig. 1.

3.2. None of the suppressor mutations is in bimC

Our genetic analysis on these strains was first focused on testing whether any of them represents an intragenic suppressor, which contains a *bimC* mutation compensating for the structural/-functional defect caused by the *bimC*4 mutation. To do this, we crossed every *sbc*#/*bimC*4 (# indicates a *sbc* number such as 1 and 2) strain to another strain containing the wild-type *bimC* gene. We reasoned that if the suppressor mutation is in *bimC*, then we should not obtain any progeny with a *bimC*4 phenotype at 42 °C. However, every cross produced progeny with a *bimC*4 phenotype. Thus, none of the suppressor mutations is in the *bimC* gene. From

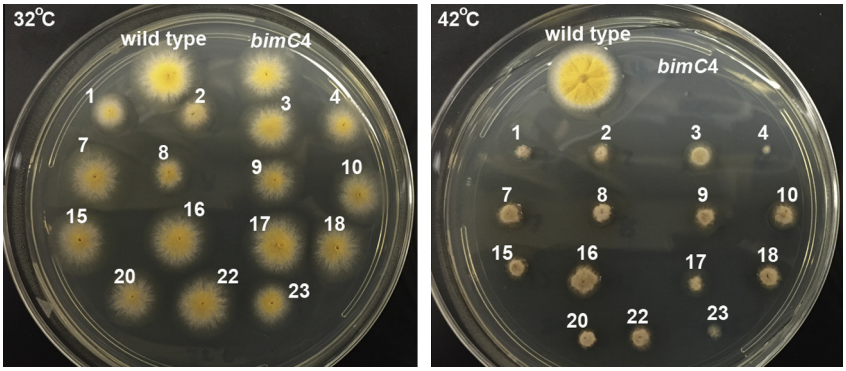

Fig. 1. Colony phenotypes of the *bimC*4 suppressors at 32 °C (permissive temperature for *bimC*4) and 42 °C (restrictive temperature for *bimC*4). The strains were grown for 2 days on YAG plates. Every suppressor strain contains the *bimC*4 mutation plus the *sbc* (suppressor of *bimC*4) mutation but labeled only with a number for simplicity (the number corresponds to the *sbc* number). At 32 °C, the *bimC*4 mutant grows as well as the wild-type control. At 42 °C, the *bimC*4 mutant is not viable. The *sbc* mutations made the *bimC*4 mutant viable so that the suppressors strains formed small colonies. All the original suppressor strains are shown except for 4 and 20, which were progeny from a cross. The extent of growth shown here does not necessarily indicates the strength of suppression because an original suppressor strain may contain other non-related mutations that affect growth.

every cross, we were also able to see a class of progeny that grew like the original suppressor strains, and these should have the genotype of *sbc#/bimC*4. For sequencing analysis, we used these progeny instead of the original suppressor strains.

3.3. Thirteen out of the fifteen suppressor strains contain mutations in the klpA gene

Since *klpA* deletion suppresses the *bimC*4 lethality, it is possible that null or loss-of-function mutations in *klpA* can be isolated by this method. Thus, we directly tested if the suppressor strains carry any mutation in the *klpA* gene. Specifically, we amplified *klpA* genomic DNA in the suppressor strains with high-fidelity polymerase for sequencing analysis. From fourteen out of fifteen *sbc#/bimC*4 strains, we were able to amplify the ~3 kb *klpA* genomic DNA. However, we were not able to obtain any PCR product from the *sbc*3/*bimC*4 strain, although several different sets of primers were tried on more than one DNA preparation, and this strain was not included in further sequencing analysis. It remains possible that the *klpA* locus may be grossly altered in this strain, and our genetic analysis result is consistent with the possibility that the suppression-causing mutation is linked to *klpA*. Specifically, not a single progeny with a *bimC*4 phenotype was found after we analyzed more than 80 progeny from a cross between the original *sbc3/bimC*4 strain and a Δ*klpA* strain.

For the other fourteen *sbc#/bimC*4 strains, we performed sequencing analysis of the ~3 kb *klpA* genomic DNA. Our results show that 13 suppressors contain a mutation in the KlpA-coding region. Except for the *sbc*1/*bimC*4 strain that contains a deletion of nucleotide G causing a frame shift after aa164 of KlpA, all other 12 strains contain mutations around the region encoding the KlpA C-terminal motor domain. The nucleotide changes in *sbc*2, *sbc*4, *sbc*7, *sbc*8, *sbc*9, *sbc*15, *sbc*17, *sbc*20 and *sbc*22 cause missense mutations affecting the motor domain. Specifically, they are R408W (*sbc*15), T431K (*sbc*4), I455S (*sbc*9), F466S (*sbc*17), I510N (*sbc*8), L566S (*sbc*7), T598G (*sbc*22), L667P (*sbc*20) and L687P (*sbc*2) (Fig. 2). In addition, the *sbc*16/*bimC*4 strain contains a nonsense mutation, K548Stop, which deletes the C-terminal motor domain of KlpA. This demonstrates that null or loss-of-function mutations of *klpA* can be identified from this genetic screen. The *sbc*10/*bimC*4 and *sbc*18/*bimC*4 strains contain exactly the same mutation in *klpA*, an insertion of a nucleotide G that causes a frame shift after aa753. One possibility we cannot rule out is that the same original suppressor might have been picked twice from the original plate after mutagenesis and treated as different strains. It is interesting to point out that aa753 is only 18 amino acids away from the Stop codon, and the mutation changes VHNTHIGT AKKQTRVRDVStop to GTQHSHWNREETDPCPStop. While the amino acids affected could be specifically required for KlpA function, the possibility that they affect the overall folding/stability of KlpA is not excluded.

While we have not transformed any mutation-containing *klpA* genomic fragment into a *bimC*4 mutant to directly demonstrate suppression caused by the *klpA* mutation, our genetic analyses on several suppressor strains including *sbc*10/*bimC*4 and *sbc*16/*bimC*4 are consistent with suppression being caused by the *klpA* mutations. Specifically, not a single progeny with a *bimC*4 phenotype was found after we analyzed more than 100 progeny from a cross between a Δ*klpA* strain and an *sbc*10/*bimC*4 or an *sbc*16/*bimC*4 strain.

The *sbc*23/*bimC*4 strain is the only strain that does not contain any mutation in the KlpA-coding region or in introns of the *klpA* gene. To confirm this result, we performed the sequencing analyses on both the original suppressor and the *sbc*23/*bimC*4 progeny from the cross between the original suppressor and a wild type strain. The results of these analyses further confirmed that the *sbc*23 mutation is not in the KLPA-coding region or in introns of the *klpA* gene. We also analyzed a cross between the original *sbc*23/*bimC*4 strain and a Δ*klpA* strain and found four *bimC*4-like progeny among 75 analyzed progeny, consistent with the idea that the *sbc*23 mutation is not linked to *klpA*. However, as *sbc*23 is a very weak suppressor (Fig. 1) and the *sbc*23/*bimC*4 strain is only slightly bigger than the *bimC*4 mutant, further work will be needed to confirm this conclusion.

4. Discussion

In this work, we provided data to suggest that rescue of the *bimC*4 lethality is mainly achieved via mutations in *klpA*, the only kinesin-14-encoding gene in *A. nidulans*. Although the *bimC*4 mutation has not been fully characterized, its temperature-sensitive nature suggests that the full-length protein can be made and therefore it is possible that an alteration of BimC itself may compensate for the defect caused by the *bimC*4 mutation. Our study, however, indicates that the chance of this happening would be low based on the fact that none of the suppressor mutations we obtained is in the *bimC* gene itself. This result supports the notion that the *bimC*4 mutant is an excellent tool for identifying kinesin-14 inhibitors.

Our identification of the *klpA* kinesin-14 mutations from the genetic screen is consistent with previous data from *S. cerevisiae*

Fig. 2. Positions of the amino acids that are mutated in the *bimC*4 suppressor mutants. A sequence alignment of the motor domain of *A. nidulans* KlpA (accession CAA45887) with that of human HSET/KIFC1 (Q9BW19) is shown. The alignment was done using CLUSTALW. Residues that are identical (*), strongly similar (:) or weakly similar (.) are shown as red, green and blue letters, respectively. Note that the amino acids mutated are either highly conserved (identical or highly similar) or right next to a highly conserved amino acid. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Hoyt et al., 1993). In *S. cerevisiae*, there are two kinesin-5-encoding genes, Kip1 and Cin8, which play redundant roles (Hoyt et al., 1992). A suppressor screen using a double mutant containing *kip1*Δ and a *cin*8 ts mutation yielded seven suppressor strains all containing missense mutations affecting the C-terminal motor domain of the kinesin-14 Kar3 (Hoyt et al., 1993). It is possible that kinesin-14 may have positive regulators in vivo. In *S. cerevisiae*, the kinesin-14 Kar3 (Meluh and Rose, 1990) has two light chains, Vik1 and Cik1 (Sproul et al., 2005; Allingham et al., 2007), which play distinct roles in regulating Kar3 function during vegetative growth and mating, respectively (Page et al., 1994; Manning et al., 1999). However, these two light chains are not present in higher eukaryotes and not in *A. nidulans* either. In theory, if there exists a protein specifically required for KlpA function, our genetic screen may discover such a protein. However, if the gene encoding such a protein is of small size or if there is another gene playing a redundant role, the probability of getting a mutation in the gene during UV mutagenesis would be low. This notion is consistent with previous data from *S. cerevisiae* in which a screen for kinesin-5 suppressors yielded seven missense mutations in Kar3 but not in the Vik1 gene (Hoyt et al., 1993), although *vik*1Δ also suppresses the lethality of the *kip1*Δ/*cin*8ts double mutations (Manning et al., 1999). In this current study, we have found a suppressor mutation (*sbc*23) possibly unlinked to *klpA*, which may suggest the existence of a regulator, but future work will be needed to confirm this notion. Nevertheless, regardless of the identity of any positive regulator, if such a regulator is essential for kinesin-14 function, its inhibitors should be just as useful as kinesin-14 inhibitors. Thus, the *bimC*4-based kinesin-14 inhibitor screen we have conceived is more inclusive than the enzyme-based screen because inhibitors of the potential regulators may also be obtained.

We are aware that during the proposed inhibitor screen, some chemicals acting on targets other than kinesin-14 (or its potential regulators) may still be obtained, and thus, a secondary screen would be necessary. It is worth pointing out that while Δ*klpA* has no obvious colony phenotype, specific *A. nidulans* gamma-tubulin mutants have been found to be synthetically lethal with Δ*klpA* (Prigozhina et al., 2001), a result similarly obtained in *Schizosaccharomyces pombe* (Paluh et al., 2000). Thus, kinesin-14 inhibitors obtained from the *bimC*4-based screen should be tested in these γ-tubulin mutants to determine if they specifically kill the mutants but not wild-type strains. Ultimately, any candidate kinesin-14 inhibitors will need to be tested using mammalian cells with and without extra centrosomes to determine if they inhibit the function of HSET/KIFC1 (Kwon et al., 2008). Because the motor domain of KlpA shows a high degree of sequence similarity to that of HSET/KIFC1 (Fig. 2), we believe that the *A. nidulans*-based screen should obtain inhibitors of HSET/KIFC1. Alternatively, one can introduce the human HSET/KIFC1 gene into *A. nidulans*, and if it can functionally substitute for *klpA*, a *bimC*4 mutant containing this human gene would be ideally suited for directly identifying HSET/KIFC1 inhibitors. *A. nidulans* is a well established genetic system for studying mitosis and microtubule motors in general (Morris, 1975; Enos and Morris, 1990; O'Connell et al., 1993; Oakley, 2004; Osmani and Mirabito, 2004; Xiang and Fischer, 2004; Peñalva et al., 2012; Pantazopoulou et al., 2014; Steinberg, 2014; Egan et al., 2015; Xiang et al., 2015) and *A. nidulans*-based drug screening has been performed previously (Kiso et al., 2004; Mircus et al., 2009; Zhai et al., 2010), further supporting the feasibility of our proposed kinesin-14 inhibitor screen.

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