Evidence That an Unconventional Actin Can Provide Essential F-Actin Function and That a Surveillance System Monitors F-Actin Integrity in *Chlamydomonas*

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ABSTRACT Actin is one of the most conserved eukaryotic proteins. It is thought to have multiple essential cellular roles and to function primarily or exclusively as filaments ("F-actin"). *Chlamydomonas* has been an enigma, because a null mutation (*ida5-1*) in its single gene for conventional actin does not affect growth. A highly divergent actin gene, *NAP1*, is upregulated in *ida5-1* cells, but it has been unclear whether *NAP1* can form filaments or provide actin function. Here, we used the actin-depolymerizing drug latrunculin B (LatB), the F-actin-specific probe Lifeact-Venus, and genetic and molecular methods to resolve these issues. LatB-treated wild-type cells continue to proliferate; they initially lose Lifeact-stained structures but recover them concomitant with upregulation of *NAP1*. Thirty-nine LatB-sensitive mutants fell into four genes (*NAP1* and *LAT1–LAT3*) in which we identified the causative mutations using a novel combinatorial pool-sequencing strategy. *LAT1–LAT3* are required for *NAP1* upregulation upon LatB treatment, and ectopic expression of *NAP1* largely rescues the LatB sensitivity of the *lat1–lat3* mutants, suggesting that the LAT gene products comprise a regulatory hierarchy with *NAP1* expression as the major functional output. Selection of LatB-resistant revertants of a *nap1* mutant yielded dominant *IDA5* mutations that presumably render F-IDA5 resistant to LatB, and *nap1* and *lat* mutations are synthetically lethal with *ida5-1* in the absence of LatB. We conclude that both *IDA5* and the divergent *NAP1* can form filaments and redundantly provide essential F-actin functions and that a novel surveillance system, probably responding to a loss of F-actin, triggers *NAP1* expression and perhaps other compensatory responses.

KEYWORDS actin; algal and plant cytoskeletons; *Chlamydomonas*; latrunculin; mutation identification by sequencing

ACTIN is one of the most highly conserved proteins across the full range of the eukaryotic phylogeny (Figure 1, A and B; Hightower and Meagher 1986; Sheterline *et al.* 1999); for example, human, yeast, and higher-plant actins are all ~90% identical in sequence. This sequence conservation suggests that actin also has highly conserved and critical functions. Indeed, since the demonstration that actin is essential in nonmotile budding yeast cells (Shortle *et al.* 1982), it has

been widely presumed that it is essential for the survival and function of most, if not all, eukaryotic cells (although this appears to have been demonstrated rigorously in few, if any, other cases). Consistent with this view, actin has been shown to be involved in a diverse range of important processes, including vesicle transport and endocytosis, the determination of cell shape, cell motility, and cytokinesis (Korn 1982; Sheterline *et al.* 1999; Doherty and McMahon 2008; Balasubramanian *et al.* 2012). In all of these roles, actin is thought to function not as globular monomers ("G-actin") but as filaments ("F-actin"), and filament formation is known to be tightly regulated, prominently by actin-binding proteins such as profilin, the formins, and the Arp2/3 complex (Doherty and McMahon 2008).

Many organisms contain multiple genes that encode actins with unknown degrees of actual or potential functional overlap; for example, mammals contain six (Perrin

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Figure 1 The two *Chlamydomonas* actins and mutant alleles identified in this study. (A) Actin phylogeny. A neighbor-joining tree for 13 actins from a phylogenetically diverse group of eukaryotes was generated as described in *Materials and Methods*; the human actin-related protein Arp1 served as outgroup. The *Chlamydomonas* actins are shown in green. Numbers, bootstrap values; scale bar, evolutionary distance in substitutions per residue. For a more in-depth phylogenetic analysis of the *Chlamydomonas* and other actins, see Kato-Minoura *et al.* (2014). (B) Amino acid-sequence alignment of the *Chlamydomonas* actins (Cr IDA5 and Cr NAP1) with human skeletal α -actin (Hs α -actin) and sites of the NAP1 LatB-sensitivity and IDA5 LatB-resistance mutations identified in this study. Numbers on right, amino acid positions; #, residues of Hs α -actin involved in direct hydrogen bonding with latrunculin A (Morton *et al.* 2000); *, sites of mutations in LatA-resistant yeast mutants (Ayscough 1997); *nap1-1* to *nap1-7*, sites mutated in these LatB-sensitive mutants (see text and Table 1); numbers 3–18, sites in IDA5 mutated in the *nap1-1* suppressors (see text and Table 4), represented by the IDA5 allele numbers assigned here. Alleles 3 (N14I), 5 (M18I), 8 (P34S), 9 (P34T), 10 (I73Y), 11 (W81R), 12 (W81R), 14 (P111S), 15 (P111Y), and 17 (T188I) were represented by a single isolate apiece; alleles 6 (M18I, V19L), 13 (P111S), 16 (L187Y), and 18 (F308S) by two independent isolates apiece; allele 4 (M18I) by four independent isolates; and allele 7 (P34S) by seven independent isolates.

and Ervasti 2010), the slime mold *Dictyostelium discoideum* contains 33 (Joseph *et al.* 2008), and the plant *Arabidopsis thaliana* contains at least 10 (McDowell *et al.* 1996). Because of the difficulties that the potential redundancy of actin poses for genetic analyses, many studies of actin function have relied instead on polymerization-blocking drugs such as the cytochalasins and the latrunculins LatA and LatB. In particular, LatA and LatB are highly effective in depolymerizing most if not all actins that have been studied to date (Spector *et al.* 1983, 1989; Ayscough 1998). However, such drug studies have unavoidable ambiguity because of the possibility that not all actin family members are equally sensitive to the drug and the danger of unknown side effects.

Given this background, the unicellular green alga *Chlamydomonas reinhardtii* has presented an opportunity but also a paradox and a challenge. It has a single gene (*IDA5*) encoding a conventional actin (Figure 1, A and B), and localization studies have suggested that IDA5 may have multiple roles both during vegetative growth and during mating (Harper *et al.* 1992; Ehler and Dutcher 1998; Kato-Minoura *et al.*

1998; Kovar *et al.* 2001; Avasthi *et al.* 2014). However, electron microscopy and labeling with fluorescent phalloxin detected F-actin structures only in fertilization tubules (Detmers *et al.* 1983; Wilson *et al.* 1997; Hirono *et al.* 2003), raising the surprising possibility that IDA5 might function during vegetative growth as G-actin rather than in filaments, as originally suggested by Harper *et al.* (1992). In another surprise, a screen for mutants defective in swimming behavior and flagellar structure yielded a null mutation in *IDA5* (originally *ida5*, henceforth *ida5-1*; a frameshift resulting in a complete loss of IDA5 protein) (Kato *et al.* 1993; Kato-Minoura *et al.* 1997). This mutation has essentially no effect on cell proliferation, although it does affect intraflagellar transport, flagellar regeneration, and fertilization-tubule formation (Kato *et al.* 1993; Kato-Minoura *et al.* 1997; Avasthi *et al.* 2014). Moreover, although very high doses of cytochalasin D caused temporary shortening of the flagella (Dentler and Adams 1992), they had no effect on proliferation (Harper *et al.* 1992).

These observations have suggested that *Chlamydomonas* cell-shape determination, vesicle transport and endocytosis,

and cytokinesis might be completely independent of F-actin function. However, interpretation of this surprising hypothesis has been complicated by the presence in *Chlamydomonas* of a gene (*NAP1*) that encodes an unconventional actin (Kato-Minoura *et al.* 1998). Although *NAP1* is only ~65% identical in amino acid sequence to *IDA5* and other conventional actins (Figure 1B), phylogenetic analyses consistently cluster it together with actins rather than with Arp1-type actin-related proteins (Figure 1A; Kato-Minoura *et al.* 2014). *NAP1* is transcriptionally upregulated in the *ida5-1* null mutant (Kato-Minoura *et al.* 1998; Hirono *et al.* 2003), so that it has been speculated that it could provide actin function under these conditions. *A priori*, the extensive sequence divergence of *NAP1* from conventional actins provides a challenge for this model. Moreover, it is unclear whether *NAP1* can polymerize into filaments: no assay has been available to examine this question in *Chlamydomonas* cells, and unlike conventional actins, the protein has been reported to polymerize poorly or not at all when expressed in mammalian cells (Kato-Minoura 2011). Assessment of possible *NAP1* function has been blocked by the absence of a *nap1* loss-of-function mutant and a lack of information about the possible sensitivity of *NAP1* to latrunculins or other drugs.

In a previous study, we used the F-actin-specific probe Lifeact (Riedl *et al.* 2008) to obtain evidence that wild-type (WT) *Chlamydomonas* cells contain LatB-sensitive F-actin filaments (Avasthi *et al.* 2014). We have now further examined the effects of LatB and performed genetic analyses beginning with the isolation of LatB-sensitive mutants. Our results suggest that *IDA5* and *NAP1* can redundantly provide essential F-actin functions and that *Chlamydomonas* cells possess a surveillance system that actively responds to insults to their actin cytoskeletons through transcriptional upregulation of the actin genes. These studies provide the tools for future in-depth analysis of actin function in *Chlamydomonas*, a key microbial representative of the plant superkingdom.

Materials and Methods

Strains and growth conditions

C. reinhardtii strains CC-124 (mt[−]) and iso10 (mt⁺) (congenic to CC-124; S. Dutcher, personal communication) were the parental strains. Hygromycin- and paromomycin-resistant derivatives were obtained by transformation with PCR-amplified *aph7*⁺ and *APHVIII* genes. Mutants were either isolated in the CC-124 background and crossed to drug-resistant derivatives of iso10 or isolated directly in a drug-resistant background. *ida5-1* strains CC-3420 (mt⁺) and CC-3421 (mt[−]) were obtained from the *Chlamydomonas* Resource Center.

Cells were grown in Tris-acetate-phosphate (TAP) medium (Gorman and Levine 1965) at 21° or 24° under constant illumination at 50–100 $\mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$. LatB was purchased from Adipogen (AG-CN2-0031, lot no. A00143/G), and dilutions into TAP medium were made from a 10-mM

stock in DMSO. Paromomycin (Sigma or EMD Millipore) and hygromycin (Sigma) were used at 10 $\mu\text{g/ml}$.

Plasmid construction and transformation by electroporation

Construction of plasmids pMO459 (*P_{HSP70A/RBCS2}:Lifeact-Venus-3FLAG:AphVIII:T_{RBCS2}*) and pMO448 (*P_{HSP70A/RBCS2}:APHVIII:T_{RBCS2}*) will be described elsewhere (M. Onishi and J. R. Pringle, unpublished results). Plasmid pMO524 (*P_{HSP70A/RBCS2}:AphVIII:T_{RBCS2}*) was constructed by PCR using pMO448 as template and primers MOP654 and MOP855 (primer sequences provided in Supporting Information, File S1), followed by a self-linearization of the PCR product by the one-step isothermal assembly method (Gibson *et al.* 2009) to remove the first exon and intron of *RBCS2* from pMO448. Plasmids pMO529 (*P_{HSP70A/RBCS2}:NAP1:AphVIII:T_{RBCS2}*) and pMO531 (*P_{TUB2}:NAP1:AphVIII:T_{RBCS2}*) were constructed in two steps. First, two DNA fragments that together covered the complete *NAP1* sequence were amplified from wild-type genomic DNA using primers MOP856 and MOP719 (fragment 1) and MOP726 and MOP857 (fragment 2) and assembled with *HpaI/NdeI*-digested pMO524. From the resulting plasmid, a DNA fragment containing *NAP1:AphVIII* was excised using *HpaI* and *BamHI* and then ligated into the same sites of pMO448 and pMO508 (M. Onishi and J. R. Pringle, unpublished results). Expression cassettes were excised with *EcoRV*; after heat inactivation of the enzyme (65°, 10 min), the digests were used for transformation by electroporation as described previously (Yamano *et al.* 2013), but using CHES buffer [10 mM N-cyclohexyl-2-aminoethanesulfonic acid, pH 9.25, 40 mM sucrose, 10 mM sorbitol]. A NEPA21 square-pulse electroporator (Bulldog Bio) was used, with two poring pulses of 250 V and 150 V for 8 ms each and five transfer pulses starting at 20 V with a decay rate of 40% for 50 ms each. Transformants were selected on TAP agar + 10 $\mu\text{g/ml}$ paromomycin.

Isolation of mutants and genetic analysis

UV mutagenesis was performed essentially as described by Tulin and Cross (2014). Cells were spread on TAP-agar plates and irradiated with a germicidal lamp in a tissue-culture hood, with inocula and times of irradiation determined empirically to yield ~300 viable colonies per plate. Irradiated plates were kept in the dark at room temperature for ~18 h to prevent light-dependent repair of UV lesions, then incubated in the light at 21° for ~1 week. Colonies were picked to a 16 × 24 array with a Hudson Robotics colony picker. Grown arrays were condensed to a 32 × 48 array using a Singer RotoR replica-plating robot, then replicated to TAP or TAP + LatB (10 μM), incubated in the light at 21° for 5–7 d, and photographed using an Olympus digital camera. The resulting JPEG images were processed with custom MATLAB software for background subtraction and alignment to a 32 × 48 grid. Differential pixel intensities with and without LatB were calculated and used for the initial selection of LatB-sensitive candidates, which were then repicked using the Singer RotoR (Stinger attachment) and retested for LatB sensitivity.

Complementation, linkage analysis, and genetic crosses for strain construction were performed essentially as described previously (Dutcher 1995; Tulin and Cross 2014). Segregant growth was recorded microscopically at 100 $\times$ with an AmScope MD500 camera. In most complementation and linkage experiments, hygromycin-resistant and paromomycin-resistant strains were mated, so that mating and meiosis could be confirmed by recovery of doubly resistant diploid or haploid progeny. For bulk analysis of meiotic progeny (without tetrad dissection), unmated haploids and stable nonzygospore diploids were removed by scraping zygospore plates with a clean razor blade before inducing meiosis by incubation in the light.

Isolation of *nap1-1* revertants

TAP agar plates with $\sim 10^7$ *nap1-1* mutant cells per plate were UV-irradiated for varying amounts of time, incubated in the dark for ~ 18 h, and returned to the light as described above. Cells from the resulting lawns were resuspended, plated at $\sim 10^7$ cells per plate on LatB-TAP agar (10 μ M LatB), and incubated at 21 $^\circ$ until colonies appeared (~ 1 week). Colonies were picked and retested for LatB resistance, and the *NAP1* and/or *IDA5* transcripts were amplified from resistant colonies by RT-PCR and sequenced as described below.

Identification of mutations by Illumina sequencing of pooled mutants and bulked segregants

For identification of the causative mutations, cultures of the initial 14 mutants were combined in five overlapping pools of seven or eight mutants each, such that each mutant was present in a unique “pool pattern” of two or three pools. These pools were processed for Illumina library preparation using reagents from New England Biolabs and oligonucleotides from Illumina; the five libraries were then sequenced at the New York Genome Center to ~ 20 – $25\times$ coverage ($\sim 3\times$ coverage for each mutant in each pool; this coverage was low but proved to be sufficient for mutant detection in almost every case). The Illumina output files (.fastq) were aligned to the *Chlamydomonas* reference genome (Merchant *et al.* 2007; file Creinhardtii_281_v5.0.softmasked.fa.gz; <http://genome.jgi.doe.gov>). The Bowtie2.bam output was processed with Samtools mpileup (Li *et al.* 2009), and the mpileup output was processed with custom MATLAB software to compress the data into matrices (rows, genome positions; columns, read counts for each call). These matrices allow sensitive detection of low-frequency alternative (mutant) calls at specific positions by comparison of the five pools to libraries from parental strains. The putative mutations were filtered for quality (base-call quality at SNP position and across the read, number of mismatches compared to reference, and mapping quality; these quality features are semi-independent) to include only high-quality calls predicted to alter coding potential (either coding-sequence change or disruption of intronic dinucleotides essential for splicing) based on the *Chlamydomonas* genome annotation (Blaby *et al.*

2014) (Creinhardtii_281_v5.5: annotation_info.txt, define.txt, description.txt, gene_exons.gff3, from <http://genome.jgi.doe.gov>).

These low-frequency mutations were then assigned to the original mutant strains based on pool profile (*e.g.*, because mutant 16 contributed to pools 1, 3, and 5, and no other mutant had this pattern, a mutation found only in libraries 1, 3, and 5 was assigned to mutant 16). Because the mutagenesis conditions resulted in hundreds to thousands of mutations that could be assigned to each mutant strain, we then searched computationally for gene models mutated (according to pool pattern) in all members of each genetic complementation group (see *Results*). For all four complementation groups, exactly one such putatively causative gene model was found (with minor discrepancies due to suboptimal coverage; see *Results*). (Note that we use the term “gene model” to reflect the hypothetical status of the functional units in the genome annotation, many of which were assigned largely or entirely on the basis of computation; Blaby *et al.* 2014).

In some cases, the initial identification of the mutations putatively causing LatB sensitivity was made or confirmed by sequencing ≥ 10 bulked LatB-sensitive segregants from crosses of mutant to wild type, as described by Tulin and Cross (2014).

Sanger sequencing and allele-specific PCR

For the seven mutants assigned to the *lat4* (*i.e.*, *nap1*) complementation group, confirmation (two cases) or initial identification (five cases) of the putatively causal mutations was performed by PCR amplification of *NAP1* using primers MOP874 and MOP721 followed by Sanger sequencing (Table 1). To search for mutations in *NAP1* and *IDA5* in the *nap1-1* phenotypic revertants, total RNA was extracted from cells treated with 10 μ l LatB for ~ 2 hr, and the transcripts were amplified by RT-PCR using primers MOP874 and MOP721 for *NAP1* and MOP875 and MOP876 for *IDA5*. The products were then purified and used for Sanger sequencing with the same primers.

To follow segregation of mutations in crosses, we used allele-specific PCR (Gaudet *et al.* 2009). For each mutation to be tested, three primers were designed (File S1). The MUT forward primer has a 3' end that is complementary to the mutant sequence except for a single-nucleotide mismatch at -3 from the 3' end; this primer also has 20 nucleotides of random sequence at its 5' end. The WT forward primer has a 3' end that is complementary to the wild-type sequence except for a mismatch at the same position as the MUT primer but to a different nucleotide. The common reverse primer is complementary to sequence ~ 100 nucleotides from the mutant site. The three primers were used together in a single PCR reaction using either purified genomic DNA or crude cell extract as template, and the products were analyzed by electrophoresis using 3% agarose gels in sodium borate buffer (Brody and Kern 2004). Products of 120-bp and 100-bp are expected for the mutant and wild-type alleles, respectively

Table 1 Summary of *lat* mutants isolated in this study^a

Mutant allele	Mutant isolate number	Change in DNA sequence ^b	Change in protein sequence ^c	Methods used to confirm mutation ^d	Linkage of mutation to <i>LatB</i> sensitivity ^e	Synthetic lethality with <i>ida5</i> ? ^f
<i>lat1-1</i>	8	GCC to G AC	A1083D ^{g,h}	P, B, AS	B	N.D.
<i>lat1-2</i>	15	CAG/ to C AA	Splice acceptor, intron 5	P	N.D.	N.D.
<i>lat1-3</i>	20	GAG to T AG	E1350-STOP ^g	P, AS	N.D.	N.D.
<i>lat1-4</i>	21	TCG to d CG	S159-FS-22aa-STOP ^g	P	N.D.	N.D.
<i>lat1-5</i>	24	CAG to T AG	Q1289-STOP ^g	P, B, AS	7 tetrads; B	Yes
<i>lat1-6</i>	35	GCG to d CG	A45-FS-12aa-STOP or upstream mutation ^g	P, B	B'	N.D.
<i>lat2-1</i>	9	CAG to T AG	Q55-STOP	P, B, AS	4 tetrads; B	Yes
<i>lat2-2</i>	10	CAG/ to C GG	Splice acceptor, intron 3	P, B	B	Yes
<i>lat2-3</i>	33	AAC to AT C	N304I	P	N.D.	N.D.
<i>lat3-1</i>	17	CGC AAA to CGT TAA	R326R, K327-STOP	B, AS	4 tetrads; B	Yes
<i>lat3-2</i>	19	AAG CCC to A GG ddd	K269R, P279-DEL	P, B	B	N.D.
<i>lat3-3</i>	31	CAG to T AG	Q400-STOP	P	N.D.	N.D.
<i>nap1-1 (lat4-1)</i>	16	GGG to A AG	G371K	P, S, AS	4 tetrads	Yes
<i>nap1-2 (lat4-2)</i>	18	CAG/ to C AC	Splice acceptor, intron 6	P, S, AS	4 tetrads	Yes
<i>nap1-3 (lat4-3)</i>	59 ^k	TGA to C GA	STOP-381R-27aa-STOP	S	N.D.	Yes
<i>nap1-4 (lat4-4)</i>	108 ^k	TGA to T GG	STOP-381W-27aa-STOP	S	N.D.	N.D.
<i>NAP1-5 (LAT4-5)</i>	106 ^k	GAC to G TT	D158V	S	N.D.	N.D.
<i>nap1-6</i>	113 ^k	GAT to G TT	D15V	S	N.D.	N.D.
<i>nap1-7</i>	114 ^k	/GTGCGTT to GTGCC AT	Splice donor, intron 2	S	N.D.	N.D.

^a In addition to the mutants listed, complementation and linkage analysis were used to assign mutants 54, 55, 85, 92, 104, 105, 111, and 115 to *lat1*; mutants 102, 110, and 117 to *lat2*; and mutants 45, 56, 62, 68, 101, 107, 109, and 116 to *lat3* (see Figure 4B).

^b Shown is the altered codon(s), splice-acceptor, or splice-donor sequence at the position in the gene indicated in the next column. The new base(s) is indicated by boldface type; /, the site of the splice; d, deletion.

^c Missense mutations are indicated by the change in predicted amino acid. STOP, normal stop codon or nonsense mutation; FS, frameshift; DEL, deletion. For mutations that result in additional predicted amino acids before a stop codon is encountered, the length of such sequence is indicated. Lengths of predicted wild-type products: *LAT1* (Cre10.g464550), 1547 (based on the translation start site manually assigned in this study) or 1476 amino acids (as currently annotated in Phytozome 10.3, see text for detail); *LAT2* (Cre10.g438250), 1065 amino acids; *LAT3* (Cre02.g076000), 507 amino acids; *NAP1* (*LAT4*; Cre03.g176833), 380 amino acids.

^d P, pool sequencing by Illumina; B, bulked-segregant sequencing by Illumina; S, Sanger sequencing of individual gene or transcript after amplification by PCR; AS, allele-specific PCR. See *Materials and Methods* for details and Figure 9B for an illustration of the allele-specific PCR.

^e Number of tetrads examined for cosegregation of *LatB* sensitivity and the putative *lat* mutation (as determined by allele-specific PCR) in a cross of the mutant to wild type; perfect cosegregation was seen in all tetrads examined. Note that bulked-segregant sequencing (B), where performed, also confirms the linkage of the putatively causative mutation to the *LatB*-sensitive phenotype. N.D., not determined.

^f Indicated are results of tests for synthetic lethality with *ida5-1*. See text, Figure 9, and Table 3 for details. N.D., not determined.

^g See text about the uncertainty in regard to the translation start site for this gene. The amino acid numbers shown assume that the more upstream start site is correct; if the currently annotated, downstream start site is correct, these numbers would change, and the *lat1-6* mutation would lie in the 5'-UTR.

^h The uncertainty from the original pool-sequencing results (see text) was resolved by the bulked-segregant sequencing and allele-specific PCR.

ⁱ Bulk-segregant reads from 10 mutant segregants had one wild-type and 34 mutant reads at the indicated position, which is embedded in an ~0.3-Mb region containing three other uniformly read noncoding SNPs. Thus, the one wild-type read is presumed to be an Illumina sequencing error, as it would otherwise require a gene conversion or a double crossover in a very small region.

^j The mutational alteration could be a point mutation or a deletion of one AC pair from an ACAC...AC run in the intron just upstream of the acceptor. Either possibility would result in a loss of the splice-acceptor sequence.

^k These mutants were not part of the original set of 14 on which pooled sequencing was performed but were assigned to *NAP1* (*LAT4*) by complementation and linkage analysis and then analyzed by Sanger sequencing.

(see Figure 9B). Purification of genomic DNA was done using the DNeasy Plant Mini kit (Qiagen) following the manufacturer's instructions. Crude cell extracts were prepared by adding ~2.5 μ l of packed cells to 50 μ l of 10 mM EDTA, pH 8.0, incubating at 105° for 15 min, and centrifuging at 12,000 $\times$ g for 1 min to remove cell debris; 1 μ l was then used for the PCR reaction. Both methods worked equally well for the allele-specific PCR.

Analysis of gene expression

Total RNA was extracted from frozen cells by adding to the frozen pellet, in the following order and without mixing: ~400 μ l acid-washed glass beads, 500 μ l of phenol-chloroform plus 500 μ l of extraction buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, 0.2 mM NaCl, 0.2% SDS), followed by vigorous

vortexing and centrifugation. RNA was then precipitated from the aqueous phase with 1 ml of ethanol, dissolved in 50 μ l of distilled H₂O, and further purified using the RNeasy Mini kit (Qiagen) following the manufacturer's instructions. [This protocol, based on one developed for yeast RNA (Cross and Tinkelenberg 1991), gave RNA preparations of greater integrity than we obtained with Trizol extraction.] A total of 0.5 μ g of total RNA was used for reverse transcription using the Maxima RT-PCR First Strand cDNA Synthesis kit (Thermo Fisher Scientific; for Figure 3) or the iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad; for Figure 6), following the manufacturers' instructions.

For evaluation of transcript levels by electrophoresis, the cDNA was subjected to PCR using primer sets (File S1) specific for *IDA5* (MOP875 and MOP876; from 100 nucleotides

Figure 2 Continued proliferation and formation of latrunculin-resistant F-actin-like structures by *Chlamydomonas* cells treated with LatB. (A) Loss and then recovery of F-actin-like structures during LatB treatment of wild-type cells. Cells of a Lifeact-expressing transformant of strain CC-124 were grown to exponential phase in liquid TAP medium at 24°, LatB was added (from a stock solution in DMSO) to a final concentration of 10 μ M, and cells were observed at the indicated times. (B) Resistance of the recovered F-actin-like structures to a high concentration of LatB. Cells from A, 120 min, were washed twice with TAP, resuspended in TAP containing 100 μ M LatB at 24°, and observed after 10 min. (C) Continued proliferation of LatB-treated wild-type cells. Cells of strain CC-124 growing exponentially in liquid TAP medium at 24° were diluted to 10⁶ cells/ml and incubated further at 24°. At time zero (arrow), LatB was added as in A; a control culture received the same concentration of DMSO alone. Cells were then counted at the indicated times. Means $\pm$ SDs from three counts are shown. (D) LatB-resistant F-actin-like structures in cells of the *ida5-1* null mutant (see Introduction). Cells of a Lifeact-expressing transformant of strain CC-3421 growing exponentially in liquid TAP medium at 24° were treated with 100 μ M LatB and observed after 10 min. Bars for A, B, and D: 5 μ m.

upstream of the start codon to 86 nucleotides downstream of the stop codon) or *NAP1* (MOP874 and MOP721; from 100 nucleotides upstream of the start codon to 115 nucleotides downstream of the stop codon) followed by agarose-gel electrophoresis.

For qPCR, the same cDNA was used with gene-specific primers (File S1) and the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) in a CFX384 Touch Real-Time PCR Detection System (Bio-Rad) with the following parameters: an initial denaturation at 95° for 30 sec, then 40 cycles of 95° for 15 sec and 55° for 15 sec. Melting-curve analysis was performed using the instrument's default setting to verify amplification of single DNA species. For each analysis, the expression levels of *IDA1* and *NAP1* were normalized first

against an internal reference and then to the value obtained for wild-type cells in the absence of LatB treatment. All reactions were performed in triplicate with at least two biological replicates or repetitions. For a control gene, we used *NUOB13* (*Cre13.g568800.t1.2*; NADH:ubiquinone oxidoreductase 18-kDa subunit), because NormFinder analysis (Andersen *et al.* 2004) of a preliminary RNA-seq dataset for wild-type cells treated with LatB indicated that it was expressed at an essentially constant level independent of LatB treatment (data not shown).

Fluorescence microscopy

Live Lifeact-Venus-expressing cells were mounted on a thin pad of TAP medium containing 1–1.5% low-melting

Figure 3 LatB-induced upregulation of *NAP1* and *IDA5* expression. Wild-type strain CC-124 and the *ida5-1* strain CC-3421 (the 1-bp *ida5-1* deletion disrupts *IDA5* translation but not mRNA formation) were grown to exponential phase in liquid TAP medium at 24°. The wild-type cells were treated with 20 μM (A) or 10 μM (B) LatB as in Figure 2 for the times indicated. (A) Total RNA was extracted and used to analyze the expression of *NAP1* and *IDA5* by RT-PCR with primers that amplify the corresponding ORFs (see *Materials and Methods*). Samples of the total RNA preparations used for RT-PCR were also analyzed to confirm their similarity in quantity and quality. (B) Total RNA was extracted and used to analyze the expression of *NAP1* and *IDA5* by RT-qPCR using gene *NUOB13* as an internal normalization standard (see *Materials and Methods*). Relative expression values were normalized to the values for wild-type cells at time zero. Means ± SDs of two independent experiments are shown.

agarose (SeaPlaque; FMC Corporation) and sealed with a coverslip. The cells were observed using a Nikon Eclipse 600-FN microscope equipped with an Apochromat ×100/1.40 N.A. oil-immersion objective lens, an ORCA-2 cooled CCD camera (Hamamatsu Photonics), and Metamorph version 7.5 software (Molecular Devices). Images were post-processed using ImageJ (National Institutes of Health) and Photoshop (Adobe) software. Images from a single experiment with a single strain were processed identically and can be compared directly. However, because of the variable expression levels of Lifeact-Venus among strains and cultures, the brightness of images cannot be directly compared across different strains or experiments.

Phylogenetic analysis and sequence comparisons

A phylogenetic tree for 13 actin proteins and human ARP1 was generated by a neighbor-joining algorithm and a bootstrap test with 10,000 iterations using a MUSCLE alignment file as input in the Geneious software version 7.0.3 (Kearse *et al.* 2012). The sequences used a wide phylogenetic spectrum: AAA51577 (*Homo sapiens*), XP_001704653 (*Giardia lamblia*), NP_116614 (*Saccharomyces cerevisiae*), XP_001699068 (*C. reinhardtii* IDA5), AAA34243 (*Volvox carteri* IDA5), XP_001703266 (*C. reinhardtii* NAP1), XP_002946759 (*V. carteri* NAP1), NP_187818 (*Arabidopsis thaliana*), AAA30152 (*Trypanosoma brucei*), P10992 (*Tetrahymena thermophila*), XP_002369663 (*Toxoplasma gondii*), P27132 (*Naegleria fowleri*), CBJ30601 (*Ectocarpus siliculosus*), and NP_005727 (*H. sapiens* ARP1).

Alignment and shading of amino acid sequences were done using Clustal Omega (Sievers *et al.* 2011) and Boxshade 3.21 (http://www.ch.embnet.org/software/BOX_form.html).

Strains, plasmids, plasmid sequences, Illumina sequencing data, and MATLAB code are available upon request. File S1 contains sequences of all DNA oligonucleotides used in this study.

Results

Upregulation of *NAP1* expression and formation of latrunculin-resistant F-actin-like structures in LatB-treated cells

One possible explanation for the surprising absence of a growth phenotype in the *ida5-1* null mutant is that the upregulated expression of *NAP1* in the mutant (Kato-Minoura *et al.* 1998) allows this unconventional actin to provide any essential actin functions (see Introduction). In that case, it might be possible to investigate actin function by treating wild-type cells with LatB, a potent inhibitor of polymerization for most if not all actins that have been tested to date (see Introduction). In short-term experiments, LatB was effective in depolymerizing the Lifeact-detected filaments, allowing clarification of the role of actin in regulation of flagellar length (Avasthi *et al.* 2014; Figure 2A, 10 min). Surprisingly, however, Lifeact-detected structures reappeared after 30–60 min of drug treatment (Figure 2A), and these structures were resistant even to a 10-fold higher concentration of LatB (Figure 2B). Correspondingly, LatB had little if any effect on the proliferation of wild-type *Chlamydomonas* cells (Figure 2C). These observations might be explained if LatB treatment induces (over)production of one or more actin-binding proteins whose interaction with IDA5-based filaments stabilizes them against the effects of the drug. However, examination of the *ida5-1* null mutant revealed the presence of F-actin-like structures that were also largely resistant even to a high concentration of LatB (Figure 2D).

To explain these results, we hypothesized that LatB-treated wild-type cells, like *ida5-1* mutant cells, upregulate *NAP1*; *NAP1* might then form LatB-resistant F-actin-like structures. Indeed, we found an ~70-fold upregulation of *NAP1* in LatB-treated wild-type cells, considerably higher than that observed in *ida5-1* mutant cells (Figure 3, A and B; note log scale in B). Interestingly, the expression of *IDA5* was also upregulated ~5-fold in the LatB-treated wild-type cells

Figure 4 Isolation and complementation analysis of LatB-sensitive (*lat*) mutants. (A) The wild-type strain CC-124, the *ida5-1* strain CC-3421, and eight of the *lat* mutants were streaked on TAP plates with and without 10 μ M LatB and incubated at 24° for 3 days. (B) Complementation analysis. Paromomycin-resistant mt+ clones (columns) and hygromycin-resistant mt- clones (rows) of the indicated *lat* mutants were analyzed as described in *Materials and Methods*. +, growth of vegetative diploid cells in the presence of 10 μ M LatB (complementation); –, no growth (noncomplementation); blank, not determined. (C) Representative results of complementation analyses. Left, growth of all vegetative diploid cells on TAP plates containing paromomycin and hygromycin (10 μ M each), confirming successful mating. Right, complementation between mutations in different genes.

(Figure 3, A and B), suggesting that *Chlamydomonas* has a regulatory system that attempts to compensate for damage to its F-actin cytoskeleton by upregulating the production of both its conventional and unconventional actins.

Isolation and initial genetic analysis of LatB-sensitive mutants

The observations described above suggested that actin functions can be carried out redundantly by IDA5 and NAP1, as speculated previously (Kato-Minoura *et al.* 1998; see Introduction). However, the absence of any means to inactivate NAP1 has prevented a direct test of this idea. If actin function is essential for *Chlamydomonas*, then a screen for LatB-sensitive mutants might yield mutations in genes specifically essential for NAP1 expression or function (including NAP1 itself). Because NAP1 is expressed little or not at all in wild-type cells (Figure 3A), a mutation eliminating its function should have little or no effect in cells not treated with LatB.

Accordingly, we mutagenized wild-type *Chlamydomonas* cells with UV as described previously (Tulin and Cross 2014) and screened mutants for inability to proliferate on agar medium containing 10 μ M LatB (see *Materials and Methods*). An initial screen yielded 14 strongly LatB-sensitive (“*lat*”) mutants. These mutants had no significant proliferation defect in the absence of LatB (Figure 4A, left) but failed to grow on 10 μ M LatB (Figure 4A, right) and displayed at least partial sensitivity to 3 or 1 μ M LatB, whereas wild-type cells could grow even on 100 μ M LatB (data not shown). The mutants were insensitive to 0.1% DMSO (the LatB carrier) and showed little or no alteration in sensitivity to other drugs (anisomycin, rapamycin, amiprofos-methyl; data not shown), suggesting that the *lat* mutants have alterations specifically affecting LatB response.

We crossed the 14 mutants to wild type and analyzed tetrads. In each case, LatB sensitivity segregated 2:2, indicating that a single genetic locus was responsible for the

Figure 5 Structures of LAT1, LAT3, and LAT2 proteins. Lengths of the proteins (see text in regard to the length of LAT1), boundaries of the identified functional domains, and positions of the mutations identified in this study (Table 1) are indicated. (A) LAT1/Cre10.g464550, with an STKc_MAP3K-like protein-kinase domain (NCBI accession cd13999, $E = 6.23 \times 10^{-54}$). (B) LAT3/Cre02.g076000, with an STKc_AMPK-like protein-kinase domain (NCBI accession cd14003, $E = 1.77 \times 10^{-65}$). (C) LAT2/Cre10.g438250, with a conserved Vca/Cre-family domain (detected by BLAST on the Phytozome website with the default search parameters). Also shown are a neighbor-joining phylogenetic tree of the *Chlamydomonas* and *V. carteri* protein family containing this domain (generated by the EMBL ClustalOmega server, <http://www.ebi.ac.uk/>) and the genomic structure of chromosome X near the LAT2 locus (image from Phytozome JBrowse) showing four of the *Chlamydomonas* paralogs in close proximity.

phenotype. Complementation tests carried out on these mutants and others isolated subsequently indicated that they defined four complementation groups (Figure 4, B and C): *lat1* (14 mutants), *lat2* (7 mutants), *lat3* (11 mutants), and *lat4* (6 mutants). In most cases, we confirmed by testing bulk meiotic products that noncomplementing mutant pairs also yielded only LatB-sensitive haploid meiotic progeny, whereas complementing mutant pairs yielded a mix of LatB-resistant and sensitive haploid progeny. One additional LatB-sensitive mutant was strongly dominant (data not shown), precluding complementation analysis. The causative mutation was found to be closely linked to *LAT4* based on a lack of LatB-resistant segregants after crosses to *lat4-1* (mutant 16) and *lat4-2* (mutant 18) testers, and sequencing subsequently showed that the strain indeed contained a *LAT4* mutation (see below).

Tetrad analysis indicated that *lat1*, *lat3*, and *lat4* are all unlinked to each other, whereas *lat1* and *lat2* are linked at a distance of ~ 18 cM [9 parental ditype (PD):0 nonparental ditype (NPD):5 tetratype (T), significantly different from the expectation of PD = NPD for unlinked genes; $P < 0.003$ by chi-square], placing them on the same chromosome, but presumably separated by several megabases, given the genome average of 10 cM/Mb (Merchant *et al.* 2007). These crosses generated the double *lat* mutants (confirmed by complementation testing) at the expected frequencies. The double mutants had no significant growth defects in the absence of LatB, and LatB sensitivity was approximately similar for double and single mutants (data not shown). These results suggest that the four *LAT* genes contribute largely or entirely to a single LatB-resistance pathway and that this pathway is essential in the presence of LatB but dispensable under normal conditions.

Taken together, these data suggest that the screen was close to saturation, such that most or all genes and pathways required specifically for cell proliferation in the presence of 10 μ M LatB have been identified.

Identification of *NAP1* and three novel *LAT* genes as sites of the *lat* mutations

The mutagenesis conditions used result in up to 100 coding-sequence changes in each survivor (Tulin and Cross 2014). To identify the specific causative mutations leading to LatB sensitivity, we used Illumina sequencing on DNA from overlapping pools of the original 14 mutants, where each mutant was represented in a distinct set of two or three pools. All mutations specific to a given mutant should then be detected as low-frequency alternative calls in a unique pattern of pools (see *Materials and Methods*). Indeed, most such mutations did align with one of the predicted pool patterns (data not shown).

Of the many mutations aligning with a particular pool pattern, only one was expected to be causative, because LatB sensitivity was in each case due to a single locus (see above). To identify these causative mutations, we used the gene assignments obtained by complementation and linkage testing. A random, noncausative mutation should almost always fall into a gene model that was hit only in a single mutant strain, whereas a mutation responsible for the LatB-sensitive phenotype should fall into a gene model that was hit in each mutant assigned to a specific complementation group. Following this logic, we tentatively identified *LAT1* as *Cre10.g464550* [five of six *lat1* mutants contained mutations that would change the coding sequence in this gene model, although in one case (mutant 8, Table 1), it was necessary to assume that one pool had failed to yield the alternative read

Figure 6 Loss or reduction of LatB-induced *NAP1* expression in *lat1*, *lat2*, and *lat3* mutants. Exponentially growing cells of the indicated genotypes were analyzed after culture for 2 hr at 24° in the absence (–) or presence (+) of 10 μ M LatB. (A) Transcript levels were analyzed by RT-PCR as in Figure 3A. *TUB1/TUB2* (the primer set used should amplify both ORFs) was included as loading control. (B) Transcript levels were analyzed by RT-qPCR as in Figure 3B. Two biological replicates were examined for each genotype; means $\pm$ SDs are shown. A cutoff of 0.5 was used because lower values were outside the linear detection ranges of the probes used and therefore not meaningful.

due to stochastic error (see Discussion)], *LAT2* as *Cre10.g438250* (three of three mutants), *LAT3* as *Cre02.g076000* (two of three mutants), and *LAT4* as *NAP1/Cre03.g176833* (two of two mutants) (Table 1). The sixth putative *lat1* mutant (mutant 35; *lat1-6*) did not show a predicted coding-sequence mutation in *Cre10.g46455*, but a single-nucleotide deletion was detected with the correct pool pattern in what is currently annotated as the 5'-untranslated region of this gene model (Table 1). It seems likely that this mutation is actually an N-terminal frameshift in the coding sequence, because there is an in-frame ATG without intervening stop codons in the 5'-untranslated region as currently annotated. However, we cannot yet rule out the possibility that the mutation is indeed in the 5'-untranslated region but has a deleterious effect on translation. In the third *lat3* mutant (mutant 17; *lat3-1*), a mutation in *Cre02.g076000* that was missed in the pool sequencing was identified subsequently by bulked-segregant sequencing (see Materials and Methods; Table 1). The $\sim$ 3.5-Mb separation of *Cre10.g464550* and *Cre10.g438250* on chromosome X is approximately consistent with the $\sim$ 18-cM genetic distance determined for *lat1* and *lat2* by tetrad analysis with a small number of tetrads (see above).

We used three different approaches to confirm the identification of the causative mutations (Table 1): bulked-segregant sequencing, PCR amplification and Sanger sequencing of the gene of interest, and an allele-specific PCR strategy (see Materials and Methods). Sanger sequencing was also used to show that all six mutants assigned to the *LAT4/NAP1* complementation group indeed carried *NAP1* muta-

tions (as did the dominant *NAP1-5* mutation) (Table 1). Bulked-segregant sequencing and allele-specific PCR on tetrads also confirmed that the putatively causative mutations cosegregated with LatB sensitivity (Table 1).

The mutations identified include at least one presumptive null allele for each gene (Figure 1; Figure 5; Table 1), including premature stop codons and/or mutations in the essential /GT...AG/ splice-site dinucleotides. Other mutations caused amino acid substitutions that were ranked as "severe" because of Blosom62 (Henikoff and Henikoff 1992) scores ≤ -2 . For *LAT1* and *NAP1*, the missense mutations also altered residues that are conserved between the predicted *C. reinhardtii* and *A. thaliana* proteins based on BLAST alignment. (We identified no missense mutations in *LAT3*, and the *LAT2* product could not be aligned to any *Arabidopsis* protein.) In a broad range of *Chlamydomonas* Ts⁻ lethal mutants, the causative mutations were up to 100 times more likely than random "passenger" mutations to be severe mutations in residues conserved in alignments to *Arabidopsis* (Tulin and Cross 2014); the findings with the *LAT* mutations confirm and extend these findings.

Mutations in the splice-site /GT...AG/ dinucleotides almost always eliminate use of the splice junction, but alternative splice junctions are often utilized instead (Brown 1996). Thus, as the splice-site mutation in *nap1-2* was the strongest candidate for a null allele of *NAP1* (Figure 1B; Table 1), we examined its protein-coding consequences by amplifying the *nap1-2* transcript using RT-PCR with primers that anneal at the translation start site and in the 3'-UTR (165 nucleotides downstream of the normal stop

Figure 7 Rescue of LatB sensitivity of *lat* mutants by constitutive *NAP1* expression. (A) Diagram of the expression constructs used in these experiments. The pMO524 construct expresses *APHVIII* (paromomycin resistance) from the strong *P_{HSP70A/RBCS2}* promoter. The pMO529 and pMO531 constructs use the *P_{HSP70A/RBCS2}* or *P_{TUB2}* promoter to express *NAP1* and *APHVIII*, connected by a short junction sequence, as a single bicistronic mRNA. See *Materials and Methods* for more details. (B) Rescue of the LatB sensitivity of a *nap1-2* mutant by constitutive *NAP1* expression. A *nap1-2* strain was transformed with the constructs excised by *EcoRV* digestion from the indicated plasmids (see *Materials and Methods*), and 32 randomly chosen paromomycin-resistant transformants for each plasmid were spotted on TAP plates without (top) or with (bottom) 5 μ M LatB. The red square indicates the strain used in C. Similar results were obtained with a *nap1-1* mutant (data not shown). (C) Partial rescue of the LatB sensitivity of *lat1*, *lat2*, and *lat3* mutants by constitutive *NAP1*

expression. The *mt- nap1-2* (pMO531 construct) strain indicated in B was crossed with *mt+ lat1-5*, *lat2-1*, and *lat3-1* strains, and segregants with the indicated genotypes were recovered (see Table 2). Single cells of the segregants were then placed by micromanipulation on TAP plates containing 3 μ M or 10 μ M LatB, incubated at 24° for 3 days, and imaged. At least two independent segregants of each genotype (three single cells apiece) were analyzed; representative images are shown.

codon). Sequencing demonstrated a 157-nucleotide deletion after exon 6, presumably by alternative splicing using the sequence ACGCAG/GGCAUC in exon 8 as an acceptor site. This alteration should cause a frameshift and synthesis of a 269-amino acid protein that lacks 120 amino acids from the normal C terminus of the 380-amino acid *NAP1* (see Figure 1B).

Sequences of the *LAT1*, *LAT2*, and *LAT3* proteins

The 1546-amino acid *LAT1* has a protein-kinase domain near its C terminus (Figure 5A) that has relatively strong similarity to MAPKK kinases from a variety of organisms. Four of the six *lat1* mutations specifically alter this domain, suggesting that it is important for *LAT1* function. The 507-amino acid *LAT3* also has a protein-kinase domain that is altered or truncated in all three *lat3* mutants (Figure 5B). For both *LAT1* and *LAT3*, BLAST searches using query sequences outside of the protein-kinase domains found no significant similarities to proteins from other organisms and thus provided no clues to their functions.

BLAST searches using either the entire 1065-amino acid *LAT2* or portions of it as query sequences found similarities only to a small gene family in *Chlamydomonas* itself (five genes in tandem on chromosome X plus two on chromosome VI; Figure 5C) and in its close relative *Volvox* (two genes; Figure 5C). The single *lat2* missense mutation that we characterized falls within the conserved domain, although the residue affected (N304) is not itself a con-

served one. No function has previously been assigned to these genes.

Roles of *LAT1*, *LAT2*, and *LAT3* in *NAP1* induction in response to LatB

The observations that wild-type *NAP1* is required for resistance to LatB and that *NAP1* expression is strongly induced by LatB suggested the possibility that the other *LAT* proteins might be required for *NAP1* upregulation. Indeed, presumed null mutations of *LAT2* and *LAT3* eliminated detectable *NAP1* induction in response to LatB (Figure 6, A and B), and the *lat1-5* mutation reduced it by ~18-fold (Figure 6B). *nap1* mutations allowed induction by LatB to a level comparable to that seen in wild type (Figure 6, A and B). As expected from the splicing defect in *nap1-2* (see above and Table 1), the cDNA from this strain migrated faster than that from wild type, *nap1-1*, or *nap1-3* strains (Figure 6A). *IDA5* mRNA levels were also induced as in wild type in all *lat* mutants (Figure 6B). Thus, the defective *NAP1* induction in the *lat1*, *lat2*, and *lat3* mutants does not appear to be due to nonspecific transcriptional defects following loss of actin function.

If the LatB sensitivity of the *lat1*, *lat2*, and *lat3* mutants results solely from their inability to induce *NAP1*, they should be rescued by expression of *NAP1* from a constitutive promoter. To test this possibility, we made constructs with heterologous promoters expressing both *NAP1* and *APHVIII* (paromomycin resistance) from a bicistronic mRNA (Figure 7A) (M. Onishi and J. R. Pringle, unpublished results). In

Table 2 Rescue of *lat1*, *lat2*, and *lat3* mutations by a *P_{TUB2}:NAP1* construct

Cross of <i>nap1-2</i> (pMO531 construct) by	No. of tetrads examined	Phenotypes of segregants ^a				Genotypes of Paro ^R Lat ^R segregants ^b
		Paro ^R Lat ^R	Paro ^R Lat ^S	Paro ^S Lat ^R	Paro ^S Lat ^S	
<i>lat1-5</i>	7	14	0	6	8	4 <i>nap1-2</i> , 4 <i>nap1-2 lat1-5</i>
<i>lat2-1</i>	12	24	0	14	10	4 <i>nap1-2</i> , 4 <i>nap1-2 lat2-1</i>
<i>lat3-1</i>	5 ^c	13	0	8	7	4 <i>nap1-2</i> , 1 <i>lat3-1</i> , 3 <i>nap1-2 lat3-1</i>

^a Resistance or sensitivity to 5 μ M LatB (Lat^R or Lat^S) and to 10 μ g/ml paromomycin (Paro^R or Paro^S) was tested on TAP plates at 24°.

^b For each cross, the genotypes of eight Paro^R Lat^R segregants were determined by allele-specific PCR (see *Materials and Methods*). Note that in the particular *nap1-2* (pMO531 construct) transformant used in these crosses, the pMO531-derived construct was apparently inserted at a site close to the *NAP1* locus itself, resulting in the linkage observed between paromomycin resistance and *nap1-2*.

^c Two complete tetrads, one complete octad, and two incomplete octads were analyzed.

contrast to *nap1-2* cells transformed with a control construct that expresses only *APHVIII* (Figure 7B, pMO524), most paromomycin-resistant transformants obtained with the *NAP1*-expressing constructs were resistant to 5 μ M LatB (Figure 7B, pMO529 and pMO531), confirming the function of the *NAP1* transgene.

We then crossed a LatB-resistant *nap1-2* transformant to *lat1-5*, *lat2-1*, and *lat3-1* strains and analyzed tetrads for paromomycin and LatB resistance. All of the paromomycin-resistant (*i.e.*, transgene-containing) segregants were resistant to LatB at concentrations up to 5 μ M, and genotyping by allele-specific PCR showed that about half of these segregants contained the mutant *lat* alleles (Figure 7C; Table 2), indicating that ectopic expression of *NAP1* can indeed largely suppress the *lat1*, *lat2*, and *lat3* mutations. This result suggests that LAT1, LAT2, and LAT3 function in a common pathway leading to *NAP1* expression, consistent with the failure to observe synergistic LatB sensitivity in *lat* double mutants (see above). Surprisingly, however, although the suppressed mutants grew nearly as well as wild type at lower concentrations of LatB (Figure 7C, top), they grew poorly or not at all on 10 μ M LatB (Figure 7C, bottom). These observations suggest that LAT1, LAT2, and LAT3 must also have at least one function in addition to supporting the upregulation of *NAP1* expression; for example, they might be important also for the expression (or upregulation) of some other gene(s) that is required for the development of full resistance to LatB (see *Discussion*).

Dependence of LatB-resistant F-actin-like structures on *NAP1*, LAT1, LAT2, and LAT3

The disappearance, then reappearance, of F-actin-like structures upon LatB treatment of wild-type cells (Figure 2A) could represent a depolymerization of LatB-sensitive F-IDA5 filaments followed by formation of LatB-resistant F-*NAP1* filaments upon *NAP1* upregulation. In this case, treatment of the *nap1* and other *lat* mutants with LatB should result in a permanent disappearance of Lifeact-labeled F-actin-like structures. This was indeed observed (Figure 8). The *nap1-1* mutant used here is a missense mutation (G371K) in a conserved residue near the C terminus (Figure 1; Table 1); the mutant protein may be deficient in polymerization or have lost its normal LatB resistance.

Synthetic lethality of *nap1* and other *lat* mutants with *ida5*

If the primary effect of LatB in wild-type cells is indeed to depolymerize F-IDA5 filaments, and if upregulated *NAP1* rescues the F-actin functions, then the *nap1* and other *lat* mutants should also be sensitive to a genetic loss of *IDA5*. To test this, we crossed representative *lat* mutants to *ida5-1* null-mutant strains and performed tetrad analysis. In all cases, the results indicated synthetic lethality between two loci (approximately one of four segregants inviable; Figure 9A; Table 3), and allele-specific PCR and tests of LatB sensitivity indicated that the two loci were *ida5-1* and the *lat* mutation, as all viable spores were LatB-sensitive *lat* single mutants, LatB-resistant *ida5-1* single mutants, or wild type at both loci (Figure 9B; Table 3). Interestingly, although the *ida5-1 nap1-2* segregants grew little or not at all, the *ida5-1 lat1-5*, *ida5-1 lat2-1*, and *ida5-1 lat3-1* segregants appeared to grow and divide normally for 2–3 d before ceasing growth and lysing during the next 24 hr (Figure 9A; data not shown). This delayed death may reflect the presence of parental *LAT* mRNAs or *LAT* proteins (or possibly of *NAP1*) that takes some days to be degraded and thus delays expression of the mutant phenotype.

Isolation of new *IDA5* alleles as suppressors of *nap1-1*

We subjected a *nap1-1* strain to UV mutagenesis (see *Materials and Methods*) and selected phenotypic revertants capable of growing on 10 μ M LatB. We anticipated three possible types of mutation: true reversion or a compensatory second-site mutation in the *NAP1* gene itself; mutation of *IDA5* to yield an *IDA5* protein that was latrunculin resistant; or mutations of other genes that somehow enhanced LatB resistance by some parallel pathway. For an initial set of 14 LatB-resistant isolates, we used RT-PCR and Sanger sequencing to sequence the *NAP1* transcript from cells treated with 10 μ M LatB for $\sim$ 2 hr to induce *NAP1* expression. All strains still contained the original *nap1-1* GG $\rightarrow$ AA mutation (Table 1), and no new mutations in *NAP1* were found. We then used RT-PCR and Sanger sequencing to sequence the *IDA5* transcript from these 14 strains plus an additional 15 LatB-resistant isolates. In 28 of the 29 cases, one or more new mutations were found in *IDA5* (Figure 1; Table 4). In some cases, these

Figure 8 Lack of LatB-resistant F-actin-like structures in *lat* mutants. Wild-type and *lat* mutant cells expressing Lifeact-Venus were grown on TAP agar, resuspended in TAP liquid medium, and grown further for 4 hr at 24°. The cells were then treated with 10 μ M LatB and observed at the indicated times. Strains used were the Lifeact-expressing transformant of strain CC-124 (compare Figure 2, A and B) and LatB-sensitive, Lifeact-expressing segregants obtained after crossing this strain with the indicated *nap1* and *lat* mutants. Bar, 5 μ m.

mutations altered residues that are near the presumed LatB-binding site (based on observations on yeast and mammalian actins), either in the primary sequence (*IDA5-10*, *-16*, and *-17*; Figure 1B) or in the 3D structure [*IDA5-3* through *IDA5-9*; Figure 1B and Protein Data Base (PDB) 1IJJ]. LatB resistance in all of the new *IDA5* mutants was dominant (Table 4), suggesting that these mutations reduce or abolish the affinity of IDA5 for latrunculin but do not affect the protein's intrinsic polymerization or depolymerization rate. In the 29th phenotypic revertant, no mutation in *IDA5* was found, and a cross to wild type indicated that the strain contained a recessive suppressor unlinked to *nap1-1* (Table 4); this suppressor gene has not yet been identified. Taken together, these data provide strong genetic evidence that IDA5 is the

normal target of LatB (resulting in LatB sensitivity in *nap1-1* mutants). The new mutations may reduce the affinity of IDA5 for LatB, thus rendering the F-IDA5 structures resistant to the drug.

Cellular response to simultaneous inactivation of IDA5 and NAP1

Wild-type or *ida5-1* cells treated with LatB exhibited few or no persistent morphological abnormalities and proliferated with little or no delay (Figure 2; Figure 10, A and B). However, although the *lat1*, *lat2*, *lat3*, and *nap1* mutant cells grew normally in the absence of LatB (Figure 4A; Figure 10, A and B), they exhibited an essentially immediate and complete block to cell proliferation upon treatment with LatB. We

Figure 9 Synthetic-lethal interactions between *ida5-1* and *lat* (including *nap1*) mutations. (A) A representative tetrad from each of the indicated crosses is shown; single-spore-derived colonies were imaged (see *Materials and Methods*) after 3–4 days of growth at 24°. See Table 3 for further details. Genotypes of the viable segregants were determined by allele-specific PCR; WT indicates that both loci had the wild-type allele. Genotypes of the inviable segregants (in brackets) were inferred by presuming 2:2 segregation for both loci. (B) An example of segregant genotyping by allele-specific PCR. The colonies indicated by numbers 1–3 in the *ida5-1* × *nap1-2* cross (A) were analyzed as described in *Materials and Methods*. The expected sizes of the PCR products for the wild-type and mutant alleles of *IDA5* and *NAP1* are indicated.

examined this behavior more closely for the presumed null *nap1-2* allele.

The *Chlamydomonas* cell cycle is separated into a long growth phase, during which cells can increase in size by >10-fold, followed by a rapid series of cell divisions; entry into cell division, and the number of divisions carried out, is dependent on cell size (reviewed by Cross and Umen 2015). We obtained a partial synchronization of wild-type and *nap1-2* cells by inoculation into low-nitrogen medium; upon depletion of the medium, such cultures consist mostly of small newborn cells. These cells were then refed with medium containing normal nitrogen levels, initiating cell growth. Cell sizes increased with little or no cell division for ≥12 hr, and in the subsequent 12 hr, almost all cells underwent multiple division cycles. *nap1-2* cells plated on LatB early in the growth cycle (4 hr after refeeding, while cells were still small) showed little increase in cell size and no cell divisions over the succeeding 22 hr, whereas wild-type controls with or without LatB, or *nap1-2* cells without LatB, all grew and carried out multiple division cycles in this time (Figure 10A). This defect could be due to a direct requirement for IDA5 or NAP1 for division; alternatively, the cells could fail division as a secondary consequence of failing to reach a critical cell size (Cross and Umen 2015). To test this, we examined wild-type and *nap1-2* cells plated on LatB 12 hr after refeeding, when they were large and would normally divide within a few hours. Plating on LatB almost completely blocked division in *nap1-2* cells but had no effect on wild type (Figure 10B). A small minority of *nap1-2* cells did appear to enter the division cycle, but these divisions appeared morphologically abnormal and did not result in completion of division (data not shown). Flow cytometry suggested that little if any DNA replication occurred after LatB addition to *nap1-2* cells (data not shown). Taken together, these results suggest that IDA5 and NAP1 are redundantly required for cell growth; there

may also be a specific IDA5/NAP1 requirement for cell division.

Discussion

Overlapping-pool sequencing and allele-specific PCR

Several methodological aspects of this study deserve comment. First, the novel overlapping-pool sequencing strategy that we used greatly facilitated the identification of the causative mutations in our collection of LatB-sensitive mutants. Other recent studies have used high-throughput, whole-genome sequencing either of individual mutants after prior meiotic mapping (Dutcher *et al.* 2012; Lin *et al.* 2013) or of bulked segregants after backcrossing (Alford *et al.* 2013; Tulin and Cross 2014) to localize the mutations of interest to genomic regions of 0.3–2 Mb, and in many cases to identify the actual causative mutation, a major improvement over methods that had been available previously. The overlapping-pool approach offers a substantial further improvement. It does not require prior meiotic mapping or backcrossing but only the definition of complementation groups (which we facilitated by the use of introduced hygromycin-resistance and paromomycin-resistance markers to allow easy selection of diploids). It makes highly efficient (and thus economical) use of a small number of library preparations and Illumina lanes: with just five libraries, we unequivocally identified the causative mutations (against a background of thousands of irrelevant mutations) in 12 of the 14 mutants included in the original analysis. We have recently also been successful in extending the approach to a set of 32 mutants sequenced in six pools (F. Cross, K. Lieberman, and M. Breker, unpublished results)—an ~5× reduction in labor, reagents, and sequencing costs—and even further extensions may be possible.

For success with the overlapping-pool approach, high-quality sequence information (including PCR-free library

Table 3 Synthetic-lethal interactions between *ida5-1* and *lat* mutations

Cross (mt– × mt+)	Tetrads analyzed	Live:dead			Number of tetrads tested by allele-specific PCR ^a
		4:0	3:1	2:2	
WT × WT	50	50	0	0	0
WT × <i>ida5-1</i>	36	33	2	1	0
<i>ida5-1</i> × WT	25	24	0	1	0
<i>ida5-1</i> × <i>lat1-5</i>	49	13	28	6	3 PD, 4 T
<i>ida5-1</i> × <i>lat2-1</i>	51 ^b	26	4	21	4 PD, 4 NPD
<i>lat2-2</i> × <i>ida5-1</i>	8 ^b	4	1	3	4 PD, 1 T, 3 NPD
<i>ida5-1</i> × <i>lat3-1</i>	60	16	28	16	4 PD, 4 NPD
<i>ida5-1</i> × <i>nap1-1</i>	18 ^b	9	6	3	4 PD, 2 T, 2 NPD
<i>nap1-2</i> × <i>ida5-1</i>	15 ^b	5	2	8	3 PD, 1 T, 4 NPD
<i>nap1-3</i> × <i>ida5-1</i>	44 ^b	16	9	19	0

^a Classified initially on the presumption (based on the apparent synthetic lethality) that parental ditype (PD), tetratype (T), and nonparental ditype (NPD) tetrads were 4 live:0 dead, 3 live:1 dead, and 2 live:2 dead, respectively. In the indicated numbers of presumed T and NPD tetrads, all viable segregants were analyzed by allele-specific PCR; in the indicated numbers of presumed PD tetrads, either the two *Lat*^S (predicted *IDA5 lat* or *IDA5 nap1-1*) or the two *Lat*^R (predicted *ida5-1 LAT* or *ida5-1 NAP1*) segregants were analyzed by allele-specific PCR. All genotypes were consistent with the hypothesis of synthetic lethality between *ida5-1* and the *nap1* or other *lat* mutation as well as with the initial designation of tetrads as PD, T, or NPD. See Figure 9 for representative data.

^b The high percentages of ditype tetrads indicate that *IDA5*, *LAT2*, and *NAP1* are all located near their respective centromeres, a conclusion confirmed by obtaining high ditype percentages also in crosses of *lat2* by *nap1* and of either *lat2* or *nap1* by a paromomycin-resistance cassette that had fortuitously integrated near the chromosome VI centromere (data not shown).

preparation) and careful bioinformatics filtering are required, because the approach relies critically on interpretation of alternative reads that may occur only once or a few times in a given library. Indeed, one causative mutation (*lat3-1*) was initially missed in this study, whereas a second (*lat1-1*) was identified only tentatively on the assumption that the relevant mutation had been missed in one of the three relevant pools. Given that Illumina sequence information is typically considered reliable only at $\geq 10\times$ coverage, these failures presumably occurred because the $20\text{--}25\times$ sequencing coverage used (only $\sim 3\times$ coverage per mutant strain) led to occasional stochastic failures to detect the alternative read. Recurrent mutations (*i.e.*, cases in which two independently isolated mutants contain identical mutations) can also interfere with the overlapping-pool approach. Although such cases will often be deconvoluted successfully, this is not guaranteed; fortunately, each mutant in the present study had a mutation that was molecularly distinct from those in others of the same complementation group. Finally, although in the present study, we made central use of the prior establishment of complementation groups, we are currently developing related approaches for which this will not be needed.

In the present study, as in many others, it was important both to confirm the initial identifications of causative mutations and to track those mutations in the segregants from genetic crosses. For both purposes, we found that an adaptation of the allele-specific PCR method (Gaudet *et al.* 2009) was rapid, cheap, and effective.

Resolution of the *ida5/NAP1* mysteries and implications for actin structure and function

The normal proliferation of the *ida5-1* null mutant (Kato-Minoura *et al.* 1997), upregulation of the highly divergent actin *NAP1* in that mutant (Kato-Minoura *et al.* 1998), and uncertainty both about the presence of F-actin in vegetative

cells (Harper *et al.* 1992; Kato-Minoura *et al.* 1998) and about the polymerization ability of *NAP1* (Hirono *et al.* 2003; Kato-Minoura 2011) raised questions that the isolation of the *nap1* and *lat* mutants have now allowed us to answer, revealing some important and rather surprising aspects of actin structure and function. First, the inviability of the *nap1*, *lat1*, *lat2*, and *lat3* mutants either in combination with *ida5-1* or upon treatment with LatB appears to demonstrate both that actin function is essential in vegetative *Chlamydomonas* cells and that either the conventional actin *IDA5* or the highly divergent actin *NAP1* can provide the essential function(s). Moreover, in wild-type cells (where *NAP1* is not expressed), the F-actin-specific probe Lifeact detects structures that disappear rapidly upon LatB treatment, whereas in *ida5-1* cells, or in wild-type cells treated for longer times with LatB, it detects similar structures that are highly resistant to LatB and are absent both in *nap1* mutants and in the *lat* mutants (which cannot upregulate *NAP1*). Thus, it seems clear that both *IDA5* and *NAP1* form F-actin filaments and that F-*IDA5* is sensitive to LatB, whereas F-*NAP1* is resistant. It was not clear *a priori* that an actin as divergent as *NAP1* could either form filaments or provide normal actin functions, and we are not aware of other cases in which an F-actin has been shown to be resistant even to high doses of latrunculin. In addition, the isolation of *IDA5* mutations as suppressors of the LatB sensitivity of a *nap1* mutant provides a formal demonstration that F-*IDA5* is the principal or exclusive target of LatB in wild-type cells. An interesting question that remains unanswered is whether *IDA5* and *NAP1* could/would assemble into common filaments if they were both expressed in the same cells.

It is important to note that although *NAP1* can provide the essential F-actin function(s) in the absence of *IDA5*, F-*NAP1* does not appear to be functionally identical to F-*IDA5*. *ida5-1* cells have several subtle but significant defects, such as altered inner-arm dynein structure in their

Table 4 *IDA5* alleles isolated as suppressors of the LatB sensitivity of *nap1-1*

Allele ^a	Nucleotide change(s) ^b	Amino acid change(s)	No. of isolates	Dominance ^c
<i>IDA5-3</i>	AAT to ATA	N14I	1	D
<i>IDA5-4</i>	ATG to ATT	M18I	4	D
<i>IDA5-5</i>	ATG to ATA	M18I	1	N.D.
<i>IDA5-6</i>	ATG GTG to ATA TTG	M18I, V19L	2	N.D.
<i>IDA5-7</i>	CCC to TCC	P34S	7	D
<i>IDA5-8</i>	TTC CCC to TTT TCC	F33F, P34S	1	N.D.
<i>IDA5-9</i>	TTC CCC to TTT ACC	F33F, P34T	1	N.D.
<i>IDA5-10</i>	ATT to TAT	I73Y	1	D
<i>IDA5-11</i>	TGG to CGG	W81R	1	D
<i>IDA5-12</i>	TGG to AGG	W81R	1	D
<i>IDA5-13</i>	CCC to TCC	P111S	2	D
<i>IDA5-14</i>	TCC CCC to TCT TCC	A110A, P111S	1	D
<i>IDA5-15</i>	CCC to TAC	P111Y	1	D
<i>IDA5-16</i>	CTC to TAC	L187Y	2	D
<i>IDA5-17</i>	ACC to ATC	T188I	1	D
<i>IDA5-18</i>	TTC to TCC	F308S	2	D
Non- <i>IDA5</i>	Unknown	Unknown	1	r

^a Allele names start from *IDA5-3* to accommodate *ida5-1* ("*ida5*" in Kato *et al.* 1993) and *ida5-2* ("*ida5-t*" in Kato-Minoura *et al.* 1997).

^b Boldface type shows the altered nucleotides.

^c Tested by a complementation analysis between these strains and a *nap1-3* mutant. D, dominant; r, recessive; N.D., not determined.

flagella and abnormal and inefficient fertilization-tubule formation (Kato *et al.* 1993; Kato-Minoura *et al.* 1997). Moreover, although the type-VIII myosin MYO2 showed a LatB-sensitive localization like that of F-actin in wild-type cells (Avasthi *et al.* 2014), it did not show a detectable localization in *ida5-1* cells (our unpublished data), suggesting that F-NAP1 cannot serve as an efficient track for this motor. Although NAP1-like proteins have been found only in the close relatives of *Chlamydomonas* within the green algae, further study of their molecular functions (what they can and cannot do) should provide broader insights into actin structure and function.

Importantly, as the *nap1* and *lat1-lat3* mutations greatly reduce or eliminate NAP1 function, and LatB ablates *IDA5* function, we can now effectively explore the cellular roles of actin in *Chlamydomonas*. Our limited results to date (Figure 10) suggest that actin has roles both in cell growth and in cell division; future studies should be able to clarify these roles. Such clarification should have significance far beyond the understanding of *Chlamydomonas* itself, as this alga can now serve as an experimentally tractable representative not only of the plant superkingdom (where genetic studies of actin function are challenging because of the multitude of actin genes) but also of at least some fraction (Rogozin *et al.* 2009) of the vast majority of the eukaryotic world that lies outside of the opisthokonts (animals, fungi, and their close relatives), where most studies of actin function have been done. For example, is the role of actin in endocytosis, well documented in the opisthokonts (Goode *et al.* 2015), universal (and thus presumably ancestral) in eukaryotes more generally? Does the formation of cleavage furrows (which is nearly universal in eukaryotes other than higher plants) always involve actin (Balasubramanian *et al.* 2012), even though type-II myosin (a component of the opisthokont actomyosin ring) is not present in groups other than the opis-

thokonts and their "close" relatives the amoebozoa (Richards and Cavalier-Smith 2005)?

The F-actin surveillance pathway, the roles of LAT1-LAT3, and the evolution of NAP1

When F-actin is lost either by *ida5* mutation or by treatment of wild-type cells with LatB, *Chlamydomonas* cells respond by massively upregulating *NAP1* (and, to a lesser extent, *IDA5*). This surveillance pathway and response are adaptive, because the ability of NAP1 to provide essential F-actin functions and its resistance to depolymerization by LatB allow the cells to survive and continue proliferating in the face of what would otherwise be a lethal insult. Regulatory responses to actin depolymerization have been observed in other systems. In yeast, actin perturbations trigger transient G2 arrest (the "morphogenesis checkpoint") via a pathway involving activation of the MAP kinase Mpk1 and impinging on the Cdc28 phosphatase Mih1 (Harrison *et al.* 2001). Although it seems likely that changes in gene expression also result from such Mpk1 activation, this possibility does not appear to have been examined. In mammalian cells, both actin and actin-binding proteins participate in transcriptional and post-transcriptional regulation of a variety of genes in response to shocks to the actin cytoskeleton (Miralles and Visa 2006; Olson and Nordheim 2010; Aragona *et al.* 2013; Mana-Capelli *et al.* 2014). In the best-studied case, perturbations in F-actin integrity are sensed by altered abundance of free MRTF (a G-actin-binding protein), which in turn regulates the transcription factor SRF (Olson and Nordheim 2010). As in *Chlamydomonas*, these pathways regulate the expression of actin isoforms; they also regulate the expression of genes for actin-binding proteins, among others. In plant cells, disturbance of F-actin under attack by pathogens has been reported to induce genes

Figure 10 Growth defects caused by loss of F-actin. Semisynchronized cultures (see text and Tulin and Cross 2014) of wild-type (CC-124) and *nap1-2* cells were collected at 4 hr (A) or 12 hr (B) into the light phase and plated on TAP plates with or without 10 μ M LatB. The cells were then observed after the times indicated.

involved in the innate-immune response (Day *et al.* 2011; Matoušková *et al.* 2014).

LatB treatment could induce *NAP1* if F-actin represses *NAP1*, if G-actin–LatB complexes induce *NAP1*, or if normal (LatB-free) G-actin represses *NAP1*; these possibilities are not mutually exclusive. *ida5-1* mutants express *NAP1* (although at a lower level than wild-type cells treated with LatB); thus, activation of *NAP1* by G-actin–LatB complexes cannot be the exclusive mechanism. LAT1, LAT2, and LAT3 are required for *NAP1* upregulation in response to LatB, and the synthetic lethality of *lat1*, *lat2*, and *lat3* with *ida5-1* suggests, but does not prove, that the LAT proteins are also required for *NAP1* induction in the genetic absence of *IDA5*. A conditional *IDA5* mutant will be required to test this idea.

The degree to which the pathways for response to actin perturbations in other organisms resemble the surveillance pathway we have found in *Chlamydomonas* is not clear. The three LAT proteins do not have obvious orthologs in organisms beyond the closely related green algae, and their sequences have provided no clues to their function beyond the presence of serine-threonine-kinase domains in LAT1

and LAT3. The rescue of the *lat1-lat3* mutations by ectopic expression of *NAP1* indicates that the regulation of this gene is the most critical role of the LAT proteins. However, the rescue was only partial (rescue on 5 μ M LatB, but not on 10 μ M LatB), which suggests that the LAT proteins may have one or more other roles (*e.g.*, in the regulation of other genes) that are necessary for full resistance to the drug. However, we have not yet ruled out the possibility that the levels of ectopic expression were simply not sufficient for full LatB resistance. Although our screen for *lat* mutants appeared to be nearly saturated, it seems unlikely that LAT1, LAT2, and LAT3 constitute the entire F-actin surveillance pathway. In particular, at least one transcription factor would appear to be required, because none of the three LAT proteins contains a recognizable DNA-binding motif, along with at least one actin-binding protein (which might conceivably be one of the three LAT proteins). Such genes might have been missed in our screen because they are essential even in the absence of LatB or are functionally redundant.

How *NAP1* achieves latrunculin resistance and how it evolved remain unclear. One possibility is that *NAP1* may have a very low intrinsic depolymerization rate. As seen with

the yeast *act1-159* mutant (Belmont and Drubin 1998), reduced filament turnover can reduce the efficacy of G-actin-sequestering drugs like latrunculins. This idea is attractive because such an alternative actin would display resistance to multiple antiactin drugs and therefore seemingly provide the cells with a major evolutionary advantage. However, it would be surprising if a poorly depolymerizing actin could fulfill all essential actin functions, given how tightly filament turnover is regulated in other systems (Doherty and McMahon 2008). Careful biochemical experiments using purified NAP1 and *in vivo* experiments using pharmacological and genetic manipulation of actin dynamics should be able to illuminate these possibilities.

Another possibility is that NAP1 has a low affinity for the drug, as do the latrunculin-resistant mutant actins in other organisms (Ayscough and Drubin 1996; Fujita *et al.* 2003) and (probably) the mutant IDA5 proteins identified here as suppressors of *nap1-2*. Indeed, one of the five amino acid residues directly involved in hydrogen bonding between rabbit-muscle actin and LatA (Morton *et al.* 2000) is altered in NAP1 (His for Tyr at position 73: Figure 1B). This simple model would be consistent with the near-normal phenotype of the *ida5-1* mutant but would seem to require exposure of the ancestors of modern *Chlamydomonas* (and related Volvocales algae, the only other organisms in which close homologs of NAP1 have been found: Kato-Minoura *et al.* 2014) to a latrunculin-like toxin during evolution. Although the latrunculins themselves are the products of marine sponges (Spector *et al.* 1983), and *Chlamydomonas* lives in fresh water, such an exposure is certainly possible. Moreover, because the LatA-binding site in actin is located close to the ATP/ADP-binding pocket (Morton *et al.* 2000), it is possible that other, currently unknown natural toxins target the same region as do the latrunculins, thus promoting the evolution of resistant proteins with alterations in that region.

It may also be relevant to the evolution of NAP1 that modern *Chlamydomonas* also shows upregulation of IDA5 in response to the loss of F-actin during LatB treatment; this upregulation may be a vestige of an early step in the evolution of a surveillance pathway and then a toxin-resistant paralog. Controlled expression of the paralog could prevent its product from interfering with the function of normal actin in the absence of stress.

Examples of highly divergent actins can also be found in other organisms. For example, of the 10 *Arabidopsis* actins, ACT5 and ACT9 share only ~70% identity with the other, more conventional actins. Because of this sequence divergence and a lack of evidence for expression, ACT5 and ACT9 are currently considered to be pseudogenes (McDowell *et al.* 1996), but it may be that the conditions that provoke their expression, and under which their products become useful, have just not been discovered. Accelerated actin evolution under environmental pressure might be another example of the evolutionary arms races that have been found to promote very rapid evolution in proteins that otherwise

would be expected to be highly conserved (Malik and Henikoff 2001).

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Evidence That an Unconventional Actin Can Provide Essential F-Actin Function and That a Surveillance System Monitors F-Actin Integrity in *Chlamydomonas*

Masayuki Onishi, John R. Pringle, and Frederick R. Cross

Primer name	Sequence 5'-3'	Target gene	Purpose
Plasmid construction			
MOP654	gttaacgtaccaaggccttgatagCATatggacgatgcgttgctgac	AphVIII	Construction of pMO524
MOP855	tcaaggccttggtacgttaactttaagatgttgagtacttctctgtaaa	RBCS2 promoter	Construction of pMO524
MOP856	agaagtcactcaacatcttaagttaacATGACTTCGGCCTTCCAGACACTG	NAP1 genomic 5' half FW	Construction of pMO529, pMO531
MOP719	CTGTTCTGCTTGGGGTTTCGC	NAP1 genomic 5' half RV	Construction of pMO529, pMO531
MOP726	CATCTGGCGCCACACCTTTGAGGAC	NAP1 genomic 3' half FW	Construction of pMO529, pMO531
MOP857	gcaacgcatcgccatATGcTCAGAAGCACTTGCGGTGCAATGCCC	NAP1 genomic 3' half RV	Construction of pMO529, pMO531
Allele-specific PCR			
MOP861	GAACAGCAGCTCCGCGcCC	NAP1	as WTallele_FW for nap1-2
MOP862	acttgataggggattatctGAACAGCAGCTCCGCGCcCg	NAP1	as MUTallele_FW for nap1-2
MOP863	CATACACTGTCTACCCGTAATGTTTTCC	NAP1	as common_RV for nap1-2
MOP835	CGGCGGAGGAGTACAACGAGTtCGG	NAP1	as WTallele_FW for nap1-1
MOP836	acttgataggggattatctCGGCGGAGGAGTACAACGAGTgCaa	NAP1	as MUTallele_FW for nap1-1
MOP721	CTCCATGGGTCGAGAATCCTTCAC	NAP1	as common_RV for nap1-1
MOP811	CCGAGGTGCTGTTAAcCtC	IDA5	as WTallele_FW for ida5-1
MOP814	acttgataggggattatctCCGAGGTGCTGTTAACgCa	IDA5	as MUTallele_FW for ida5-1
MOP813	TTGCGGATGTCCACATCGCA	IDA5	as common_RV for ida5-1
MOP865	CTATCCGGCCCCACAGCAAGGCGtCC	LAT1	as WTallele_FW for lat1-5
MOP866	acttgataggggattatctCTATCCGGCCCCACAGCAAGGCGgCt	LAT1	as MUTallele_FW for lat1-5
MOP867	TGTTGTTGTTGCCGCAAGGCTGC	LAT1	as common_RV for lat1-5
MOP868	CTGTTCGAACTGATCACCGGGAtGG	LAT1	as WTallele_FW for lat1-3
MOP869	acttgataggggattatctCTGTTCGAACTGATCACCGGGAcGt	LAT1	as MUTallele_FW for lat1-3
MOP870	CGTCGGGCACTGGCAGCACG	LAT1	as common_RV for lat1-3
MOP871	CGTTCGGGGCCGTGTACAAtGC	LAT1	as WTallele_FW for lat1-1
MOP872	acttgataggggattatctCGTTCGGGGCCGTGTACAAcGa	LAT1	as MUTallele_FW for lat1-1
MOP873	GAAGCAAGTACGAACGGGTGCCGCT	LAT1	as common_RV for lat1-1
MOP916	CTGGCCCCACCACCGCCTaCC	LAT2	as WTallele_FW for lat2-1
MOP917	acttgataggggattatctCTGGCCCCACCACCGCCTgCt	LAT2	as MUTallele_FW for lat2-1
MOP918	TGCACGCATGGGTTCGAGCT	LAT2	as common_RV for lat2-1
MOP922	CAAGCAGTACGACGGCaGCA	LAT3	as WTallele_FW for lat3-1
MOP923	acttgataggggattatctCAAGCAGTACGACGGCgGtt	LAT3	as MUTallele_FW for lat3-1
MOP924	GCGCCAGAAGCTCTCAAGTAACGAT	LAT3	as common_RV for lat3-1
qPCR			
MOP883	TCATCAAGGAGGCGAAGGA	NUOB13	NUOB13_qPCR_FW
MOP884	TGTAGTCCACCACAGGCA	NUOB13	NUOB13_qPCR_RV
MOP887	CTCAACACATAGCTACCAGACTT	IDA5	IDA5_qPCR_FW
MOP888	AAAGCCAGCCTTCACCAT	IDA5	IDA5_qPCR_RV
MOP879	GTGCAAGCATCGCAAGTAAAC	NAP1	NAP1_qPCR_FW
MOP880	CGGAGCCATTATCGCAAACA	NAP1	NAP1_qPCR_RV
RT-PCR and Sanger sequencing			
MOP875	GGACATACAATAGAAGCCCGGCTTGAC	IDA5 cDNA	IDA5 -100_FW
MOP876	GCACCCCTGGATGGGCGGAAG	IDA5 cDNA	IDA5 +1220_RV
MOP874	CAGCAGTGA CTGTGTTTTCGGCGT	NAP1 cDNA	NAP1 -100F
MOP721	CTCCATGGGTCGAGAATCCTTCAC	NAP1 cDNA	NAP1 +1308_RV