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Ubiquitin-like Protein Hub1 Is Required for Pre-mRNA Splicing and Localization of an Essential Splicing Factor in Fission Yeast

Caroline R.M. Wilkinson,[1,2,*] Gunnar A.G. Dittmar,[1,4] Melanie D. Ohi,[1] Peter Uetz,[3,5] Nic Jones,[2] and Daniel Finley[1]

[1]Department of Cell Biology
Harvard Medical School
240 Longwood Avenue
Boston, Massachusetts 02115

[2]Cancer Research UK
Paterson Institute for Cancer Research
Christie Hospital NHS Trust
Wilmslow Road
Manchester M20 4BX
United Kingdom

[3]Department of Genetics and Medicine
University of Washington
Box 357360
Seattle, Washington 98195

Summary

Hub1/Ubl5 is a member of the family of ubiquitin-like proteins (UBLs) [1, 2]. The tertiary structure of Hub1 is similar to that of ubiquitin [3, 4]; however, it differs from known modifiers in that there is no conserved glycine residue near the C terminus which, in ubiquitin and UBLs, is required for covalent modification of target proteins. Instead, there is a conserved dityrosine motif proximal to the terminal nonconserved amino acid. In *S. cerevisiae*, high molecular weight adducts can be formed in vivo from Hub1, but the structure of these adducts is not known, and they could be either covalent or noncovalent [1, 5]. The budding yeast *HUB1* gene is not essential, but Δ*hub1* mutants display defects in mating [1]. Here, we report that fission yeast *hub1* is an essential gene, whose loss results in cell cycle defects and inefficient pre-mRNA splicing. A screen for Hub1 interactors identified Snu66, a component of the U4/U6.U5 tri-snRNP splicing complex. Furthermore, overexpression of Snu66 suppresses the lethality of a *hub1ts* mutant. In cells lacking functional *hub1*, the nuclear localization of Snu66 is disrupted, suggesting that an important role for Hub1 is the correct subcellular targeting of Snu66, although our data suggest that Hub1 is likely to perform other roles in splicing as well.

Results and Discussion

As a first step toward characterizing *hub1* in fission yeast, we created a null allele and found that *Sphub1* is an essential gene. Δ*hub1* cells arrested as elongated cells, suggesting that loss of *hub1* might be associated with defects in cell cycle control. Interestingly, Hub1 proteins from different species display more sequence identity than any other family of ubiquitin-like proteins, with 74% identity existing between fission yeast and human Hub1 sequences. The null allele could be rescued by cDNAs encoding either *S. pombe*, human, or budding yeast Hub1, indicating that the essential function of Hub1 is conserved evolutionarily (data not shown).

A temperature-sensitive allele of *hub1* was also generated. The *hub1-4* allele is a M70K missense mutation that results in growth at the permissive temperature of 30°C but does not form colonies at temperatures of 32°C and above (Figure 1A). Cultures of *hub1-4* cells did not increase in cell number upon shift to 36°C, indicating a first cycle arrest (Figure 1B, left). However, the cells continued to grow as shown by an increase in cell mass (Figure 1B, right). These cells became elongated but not to the extent that some *cdc* mutants do, suggesting that there might also be defects in cell growth (Figure 1E). Upon shifting to 36°C, the *hub1-4* cells appeared to undergo an initial G2 arrest as the numbers of binucleate and septated cells dropped. However, upon prolonged incubation, the cells leaked through this block and accumulated as binucleate, septated cells (Figures 1C and 1D), a finding that is consistent with defects in cell separation (data not shown).

In order to investigate the role of the C-terminal dityrosine motif, mutations were made in the *hub1* cDNA and their ability to rescue the lethality of the null mutant was examined. Surprisingly, the *hub1* cDNA lacking one or both of these tyrosines was still able to rescue the null allele, and cells containing the cDNA lacking both tyrosines grew at the same rate as those containing the wild-type cDNA (Figures 1F and 1G). Deleting more residues upstream of the dityrosine motif abrogated complementation. Furthermore, mutation of the tyrosines to several other combinations of amino acids still resulted in complementation (Figure 1F). These data indicate that the conserved dityrosine motif is not required for the essential role of Hub1 in fission yeast.

To gain insight into *hub1* function at the molecular level, a two-hybrid screen was performed, using constructs derived from the *S. cerevisiae HUB1* gene. This bait was screened individually against almost every open reading frame in the genome. Only one clone, encoding Snu66, was identified in two independent screens as binding strongly to Hub1. Snu66 was originally identified in *S. cerevisiae* as a novel protein present in the U4/U6.U5 tri-snRNP [6, 7]. This complex forms an integral part of the pre-mRNA splicing machinery. ScSnu66 and ScHub1 were found to interact very strongly in the two-hybrid assay (Figure 2A, left).

A homolog of Snu66 exists in the fission yeast genome. It shares 24% identity with budding yeast Snu66. The *S. pombe* homologs of Snu66 and Hub1 also interacted strongly in the two-hybrid system (Figure 2A, middle). This interaction was abrogated by the *hub1-4* mutation (data not shown). Snu66 is also conserved in higher

*Correspondence: cwilkinson@picr.man.ac.uk
[4]Present address: Max-Delbrück Centrum, Robert-Rössle Strasse 10, 13092 Berlin, Germany.
[5]Present address: Institute of Genetics, Forschungszentrum Karlsruhe, P.O. Box 3640, D-76021 Karlsruhe, Germany.

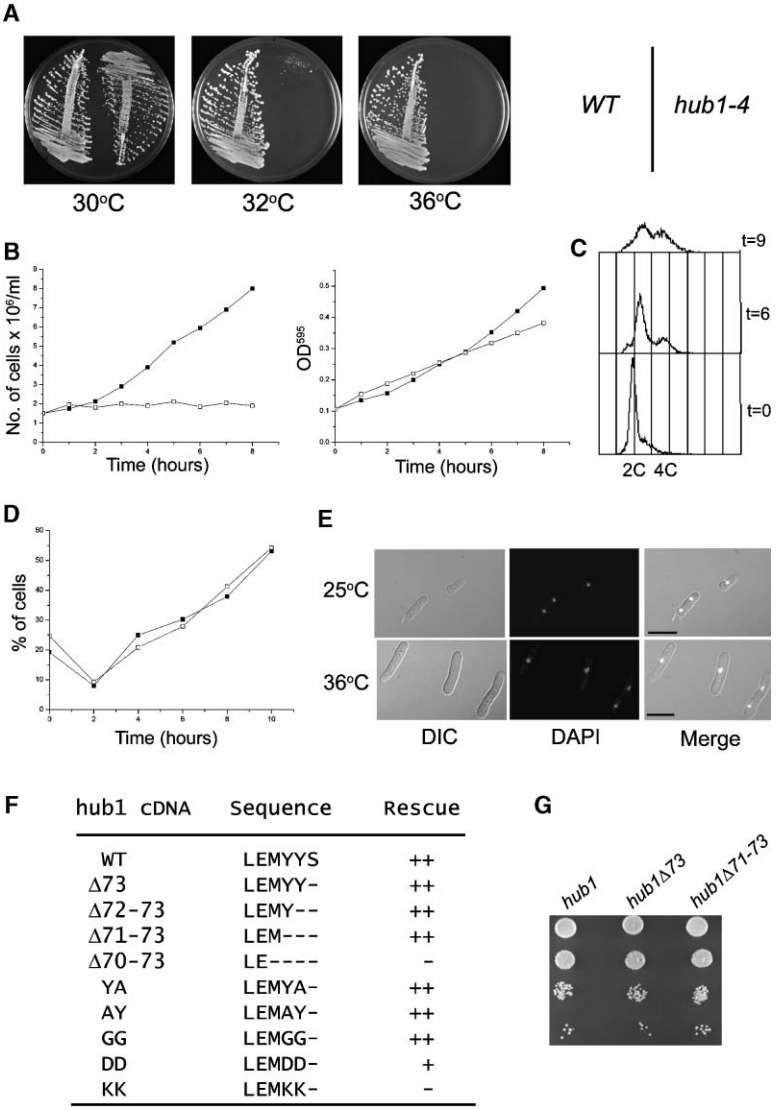

Figure 1. Loss of Fission Yeast *hub1* Activity through the *hub1-4* Temperature-Sensitive Allele, but Not Removal of the C-Terminal Di-tyrosine Motif, Results in Cell Cycle Defects

(A) Wild-type and *hub1-4* cells were streaked to single colonies on yeast extract media and incubated for 3 days.

(B) *hub1-4* cells display cell cycle defects at the restrictive temperature. Exponentially growing *hub1-4* cells were grown at 25°C or 36°C, and the numbers of cells (left) and cell mass at an OD_{595} (right) were determined. The closed and open squares indicate cells growing at 25°C or 36°C, respectively.

(C) The DNA content of *hub1-4* cells growing at 25°C (t = 0) or 36°C for 6 hr and 9 hr was determined by FACS analysis. The positions corresponding to 2C and 4C content are indicated.

(D) The percentage of binucleate (open squares) and septated cells (closed squares) were determined by staining cells with DAPI or calcofluor, respectively, at various time points after the shifting *hub1-4* cells to 36°C.

(E) *hub1-4* cells grown at 25°C and 36°C for 8 hr were stained with DAPI and photographed. The scale bar equals 10 μm.

(F) *hub1* cDNAs with either truncations (Δ) or mutations at the C terminus were assayed for their ability to rescue the *hub1* null allele when expressed from the *nmt81* promoter in the pREP81X vector in the absence of thiamine. Wild-type growth is indicated by ++, no growth by –, and growth that is slower than wild-type by +. The wild-type sequence of the C terminus is 68-73: LEMYYS.

(G) 10 μl of serial dilutions of the *hub1* null allele expressing either the wild-type *hub1* cDNA, the Δ73 mutant cDNAs, or the Δ71-73 mutant cDNAs from the *nmt1* promoter in pREP3X were plated on selective media and grown for 4 days at 30°C. The dilutions range from 10^6 cells/ml to 10^3 cells/ml.

eukaryotes. The human *SART-1* gene encodes a protein that is 21% identical to *S. pombe* Snu66. Like Snu66, SART-1 has also been found as a component of splicing complexes [8]. SART-1 interacted with UBL5, the human homolog of Hub1, in the two-hybrid system (Figure 2A, right). Taken together, these data indicate that SART-1 and SpSnu66 are the orthologs of ScSnu66 and that the interaction between Snu66/SART-1 and Hub1/UBL5 is widely conserved amongst eukaryotes. Hub1 and Snu66 were shown to interact directly by an in vitro binding assay carried out with purified recombinant proteins (see Supplemental Figure S1 available with this article online). Based on these results, and on two-hybrid assays (data not shown), the region of Snu66 that is required for interaction with Hub1 was localized to the N-terminal 107 amino acids.

To test whether the interaction between Hub1 and Snu66 is physiologically significant, we overexpressed *snu66* cDNA in the *hub1-4* mutant. Overexpression of the *snu66* cDNA was partially able to rescue the *hub1-4* mutant, as these cells acquired the ability to form colonies at 32°C (Figure 2B) although not at the fully restrictive temperature of 36°C. These data imply that the interaction between Snu66 and Hub1 may be defective in the *hub1-4* mutant cells and that overexpression of *snu66* compensates for this, resulting in partial suppression of temperature sensitivity. Attempts to coimmunoprecipitate Hub1 and Snu66 from *S. pombe* cells were not successful, possibly because the interaction between these two proteins is transient or because antibody recognition of the complex is sterically hindered. It does not seem likely that Snu66 is covalently modified by Hub1, as immunoprecipitation of Snu66 from cells that contained a tagged version of Hub1 did not allow for the identification of a high molecular weight band corresponding to a putative adduct (data not shown). The budding yeast *SNU66* gene is not essential for growth, although Δ*snu66* cells display defects in splicing at low temperatures [9, 10]. In contrast, we found that *S. pombe snu66* is an essential gene, the loss of which results in defects in pre-mRNA splicing (Figure S2).

The interaction between Hub1 and Snu66 suggested

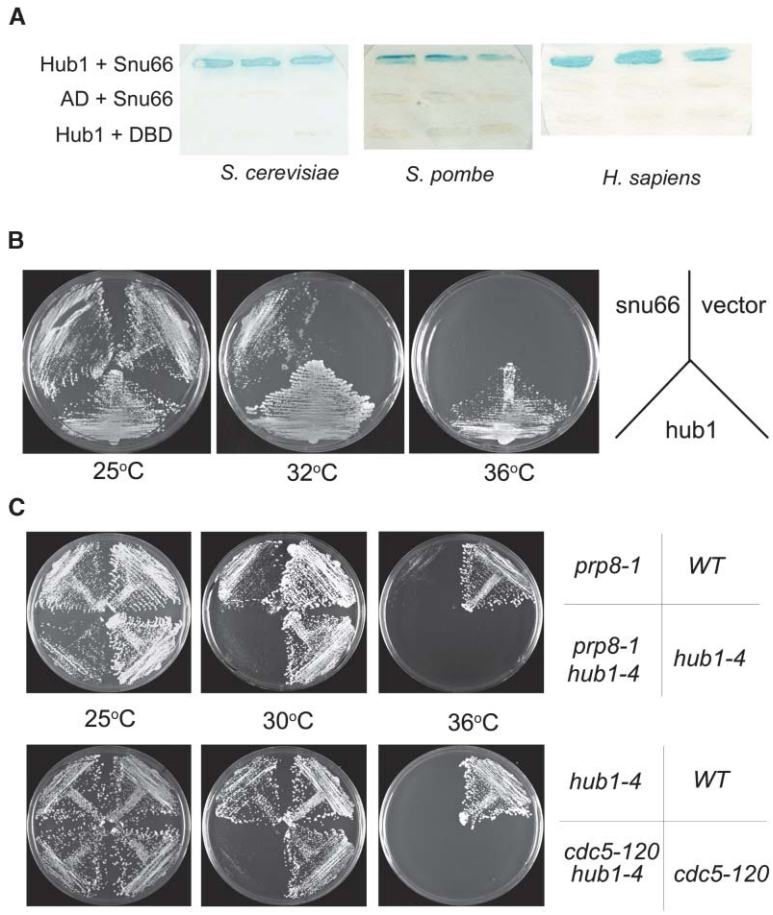

Figure 2. Hub1 Interacts Physically with Snu66 and Genetically with Both *snu66* and Other Splicing Factors

(A) Two-hybrid assay to demonstrate binding between Hub1 and Snu66. The coding sequences for Snu66 or Hub1 were fused to the DNA binding domain of LexA (DBD) or the activation domain of Gal4 (AD), respectively, and transformed into a *S. cerevisiae* host strain. Transformed colonies were patched onto selective media and subjected to filter lift β-gal assays. Blue color indicates an interaction between bait and prey proteins. The control reactions consist of either Hub1 or Snu66 fused to the AD or DBD, respectively, cotransformed into yeast with vectors containing just the DBD or AD alone. The assays were carried out using the *hub1* and *snu66* open reading frames from *S. cerevisiae, S. pombe*, or humans as indicated. SART-1 and UBL5 are the human homologs of Snu66 and Hub1, respectively.

(B) Overexpression of *Spsnu66* rescues the *hub1-4* mutant at 32°C. *hub1-4* cells transformed with either pREP3X*snu66*, pREP3X-*hub1*, or empty pREP3X vector were streaked to single colonies on selective media lacking thiamine for 5 days.

(C) The wild-type and *prp8-1*, *hub1-4*, and *prp8-1 hub1-4* mutants (top) and wild-type, *cdc5-120*, *hub1-4*, and *cdc5-120 hub1-4* mutants (bottom) were streaked to single colonies on yeast extract media at 25°C, 30°C, and 36°C for 4 days.

that Hub1 could play a role in splicing. At the same time as this study was being performed, results obtained via mass spectrometry suggested that the human homolog of Hub1 (UBL5) may be present within an intermediate form of the spliceosome [8]. We found further evidence for a role of *hub1* in splicing from synthetic phenotypes observed between *hub1-4* and other splicing mutants. The *prp8* gene encodes a member of the DEAH-box family of RNA-dependent helicases and is required for pre-mRNA splicing. *prp8-1* is a temperature-sensitive allele that confers a G2 arrest at the restrictive temperature [11, 12]. The double mutant *prp8-1hub1-4* showed a reduced restrictive temperature (Figure 2C, top), suggesting that splicing was compromised to a greater degree than in either of the single mutants. A similar synthetic effect was observed between the *cdc5-120* and *hub1-4* alleles (Figure 2C, bottom). Cdc5 is essential for pre-mRNA splicing and is found in a large snRNP-containing complex [13, 14]. These data further support the hypothesis that *hub1* has a role in pre-mRNA splicing.

The possible role of Hub1 in pre-mRNA splicing was tested directly by asking whether cells lacking functional *hub1* accumulate nonspliced transcripts. In *hub1-4* cells at the restrictive temperature, the RT-PCR procedure produced cDNA species of a size equivalent to the unspliced transcripts of the *pus1*, *sec13*, *rpa43*, and *rad24* genes (Figure 3A, compare lanes 7 and 8). Sequencing these products confirmed that they corresponded to the unspliced pre-mRNAs (data not shown). In contrast, splicing of these transcripts is apparently normal when *hub1-4* is grown at 25°C. The loss of *hub1* activity thus results in a reduction of splicing efficiency for a variety of genes (Figure 3A). This becomes apparent as soon as 1 hr after shifting to the restrictive temperature (bottom panels, compare lanes 5 and 6). As seen for *hub1-4*, unspliced transcripts also accumulate in cells expressing *hub1* from the *nmt81* promoter, after the addition of thiamine to the medium (Figure 3B, compare lanes 9 and 10). Although the defects in splicing of the above genes are not as severe as seen in the mutant *prp2-1* (Figure 3B, lane 8 and data not shown), the extent of the splicing defect seen in individual mutants may not reflect the strength of the requirement for these gene products in the wild-type cell. Also, certain genes, such as *cut6* (Figure 3C), seem to be affected to a greater extent than others. A significant amount of unspliced *cut6* was identified in samples prepared from *hub1-4* cells even at the permissive temperature (lane 9). Taken together, these data indicate that there are global defects in splicing in cells lacking functional *hub1* and that some genes are affected to a greater extent than others. The defects are not restricted to genes involved in cell cycle regulation, as nonessential genes such as *pus1* are also affected.

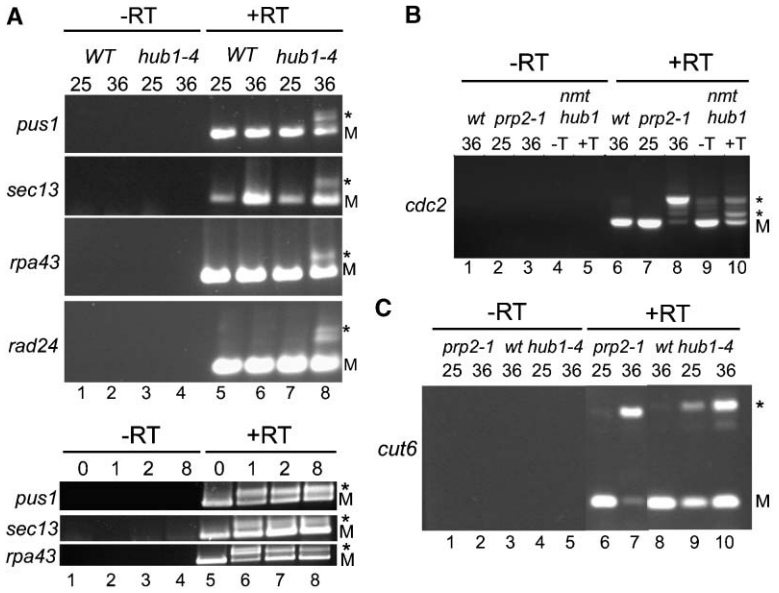

Figure 3. *hub1* Function Is Required for Efficient Pre-mRNA Splicing

(A) RNA was prepared from the *hub1-4* mutant and wild-type cells that had been grown at 25°C or 36°C for 8 hr (top panels) or for 1, 2, and 8 hr (bottom panels). cDNA was prepared and used as the template in PCR reactions with oligonucleotides complementary to various genes as indicated. The oligonucleotides were designed so that both spliced and unspliced versions of each gene could be detected. The PCR products corresponding to the mature (spliced) and (precursor) unspliced message are marked with M and an asterisk, respectively. The band below the one marked with an asterisk in the *rad24* reaction is a nonspecific product. Negative control reactions were carried out using substrate prepared from reactions carried out without reverse transcriptase (−RT).

(B) RT-PCR reactions were carried out as in (A) but using cDNA made from RNA isolated from the *nmt81hubl* strain grown in the presence of thiamine for 18 hr. RNA was also prepared from the *prp2-1* mutant grown at 25°C or 36°C for 12 hr.

(C) RT-PCR reactions were carried out as in (A) using RNA prepared from either a wild-type strain or the *prp2-1* and *hub1-4* mutants grown as described above.

We further investigated the relationship between Hub1 and Snu66 by examining the subcellular localization of Snu66 in cells lacking functional Hub1. Indirect immunofluoresence was used to visualize Snu66 in cells where the genomic copy of *snu66* had been tagged with 13 copies of the myc epitope. These cells are wild-type in growth and appearance (data not shown). Consistent with its role in splicing, we found that SpSnu66 is localized to the nucleus (Figure 4A). Strikingly, in *nmt81hub1 snu66-myc* cells grown in the presence of thiamine, this pattern of staining was severely disrupted. The Snu66 signal was found predominantly in the cytoplasm (Figure 4D), whereas in cells grown in the absence of thiamine, which are phenotypically comparable to wild-type, the signal was nuclear (Figure 4C). A similar delocalization was observed in the *hub1-4* allele when shifted to the restrictive temperature (Figure 4F), whereas the localization of Snu66 in *hub1-4* at 25°C was nuclear (Figure 4E).

To test whether loss of Hub1 results in the redistribution of other splicing factors, we examined the localization of Cdc5. Previously, Cdc5 has been shown to localize to the nucleus [13], and this pattern of localization was observed in a *hub1-4 cdc5-myc* strain grown at either 25°C or 36°C. Thus, not all splicing factors are mislocalized in the *hub1-4* strain. Furthermore, Prp3, another tri-snRNP component, appears to be nuclear in the *hub1-4* background at both 25°C and 36°C (Figures 4I and 4J). The levels of Snu66 do not appear to change in the *hub1-4* background (Figure S3), indicating that the cytoplasmic signal is not due to background staining. This role for Hub1 in controlling the nuclear localization of Snu66 is evolutionarily conserved, as shown by the loss of nuclear localization of Snu66 in the *hub1* null mutant of *S. cerevisiae* (Figure S4). These data suggest that a role for Hub1 in both *S. pombe* and *S. cerevisiae* is the correct subcellular targeting of the splicing factor Snu66.

In this study, we have characterized the *hub1* gene in fission yeast and have shown that loss of Hub1 results in defects in cell cycle progression and pre-mRNA splicing. A number of studies have identified mutations that also result in dual splicing and cell cycle defects [11–16]. Defects in pre-mRNA splicing factors presumably result in cell cycle arrest because certain key cell cycle factors have introns, and a reduction in splicing efficiency results in reduced levels of the corresponding proteins, leading to a block in cell cycle progression. In *S. pombe*, it is difficult to assign cell cycle defects in splicing mutants to inefficient splicing of a particular gene, as approximately 45% of genes have introns.

At the molecular level, we find that Hub1 interacts with a specific component of the U4.U6/U5 tri-snRNP, Snu66, and that this interaction is conserved evolutionarily. Overexpression of Snu66 suppresses the lethality of the *hub1-4* mutant. The simplest interpretation of this suppression is that elevated levels of Snu66 drive its association with mutant Hub1, resulting in enough incorporation of Snu66 into spliceosomes to support cell growth. This interpretation would imply that failing to mediate proper localization of Snu66 is the only lethal effect of the *hub1-4* mutation. However, if nuclear targeting were the only role of Hub1, addition of an exogenous NLS to Snu66 might rescue cells lacking *hub1* activity. Two versions of Snu66 tagged with an NLS at either the N terminus or C terminus were tested for their ability to rescue both a *hub1* null and *hub1-4* strain. Neither version of Snu66 was able to rescue these mutants (data not shown), implying that the Hub1-Snu66 interaction does not serve simply to localize Snu66 in the nucleus. Thus, the nuclear localization of Snu66 is

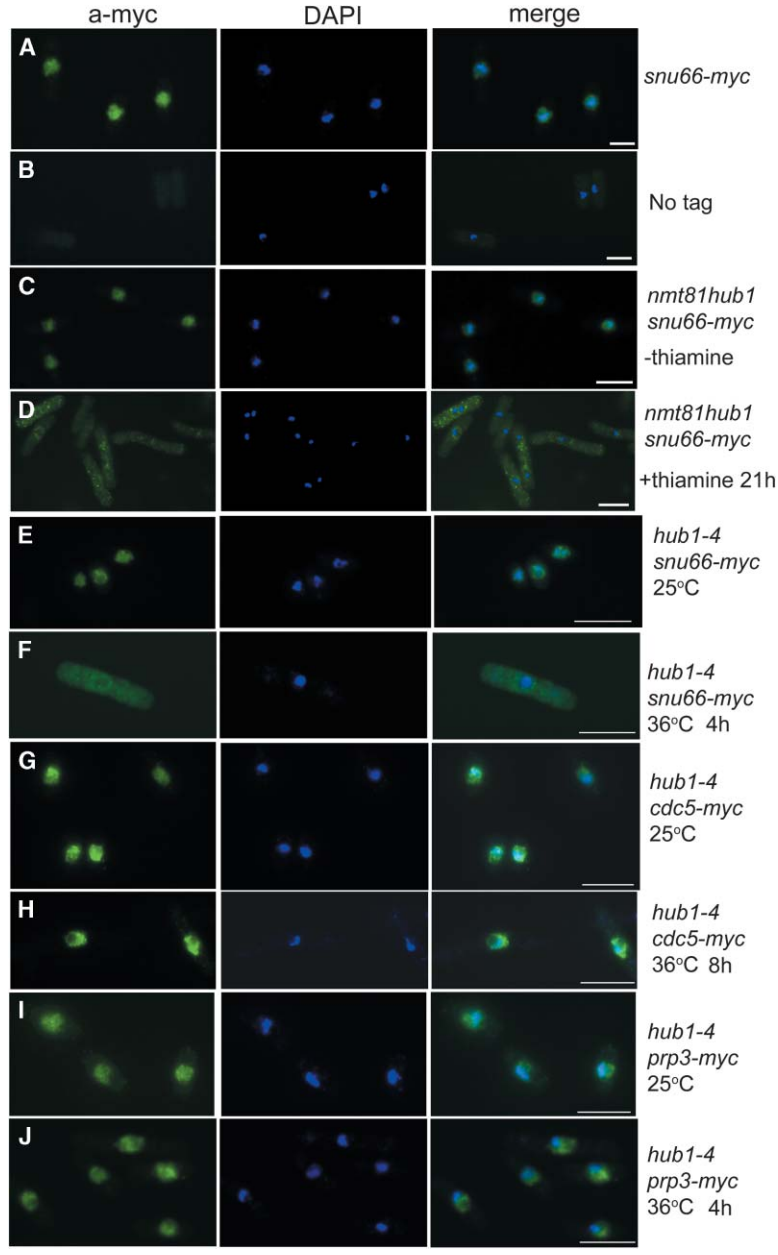

Figure 4. Loss of *hub1* Results in the Mislocalization of Snu66

Indirect immunofluoresence was carried out on cells that had been fixed with formaldehyde. The *snu66*, *cdc5*, and *prp3* genes are fused to 13 copies of the myc epitope at their C termini. Cells were stained with antibodies to the myc epitope (left) or DAPI (middle) and the resulting images merged (right). The genotypes and growth conditions were as indicated on the right side of the panels. The scale bars equal 10 μm.

dependent on Hub1 function, but the Hub1-Snu66 interaction does not function solely to effect nuclear localization. The *hub1-4* mutant displays defects in splicing after just 1 hr at the restrictive temperature (Figure 3A). At this point, however, Snu66 is still concentrated in the nucleus (data not shown), and so the earliest defect in splicing may not be due to the cytoplasmic mislocalization that is observed at later time points.

Is Hub1 a component of the spliceosome? One study found that the human homolog of Hub1 copurified with an intermediary splicing complex, B*, which is poised to catalyze splicing [8], and another has found that Ubl5 is part of spliceosomal complex B just prior to activation but after incorporation of the tri-snRNP [17]. However, in another report, functional, human spliceosomes were isolated that comprised all previously known splicing components as well as 58 newly identified proteins, but did not identify Ubl5 [18]. Purification of the yeast U4.U6/U5 tri-snRNP, including Snu66, have not identified Hub1 [6, 7]. One possibility is that Hub1 mediates some of the rearrangements that take place during spliceosome formation and that it does so via its interaction with Snu66. It is possible that Hub1 affects the tri-snRNP stability via its associations with Snu66, but this seems unlikely, as immunodepletion of the human homolog of Snu66 did not result in destabilization of the tri-snRNP [19]. Furthermore, we have found that after 1 hr at the restrictive temperature in the *hub1-4* mutant, Snu66 is still associated with the U4, U5, and U6 snRNAs, suggesting that the tri-snRNP is still intact (data not shown).

Previous studies in budding yeast have found that, in a similar manner to many ubiquitin-like proteins, Hub1

is present, in part, in complexes that are electrophoretically retarded on SDS-polyacrylamide gels [1, 5]. The high molecular weight species have been alternatively hypothesized to be covalent in nature [1], by analogy to ubiquitin-protein conjugates, or of a novel SDS-resistant noncovalent nature [5]. The present study does not distinguish between these models, because we have so far not found evidence of high molecular weight Hub1 adducts associated with its role in pre-mRNA splicing. In particular, we have not found evidence of covalent modification of Snu66 by Hub1 (C.R.M.W., unpublished data). Given the ability of C-terminally truncated Hub1 to complement the lethal phenotype of the *hub1* null mutant, it is most likely that the role of Hub1 in pre-mRNA splicing does not involve C-terminal conjugation.

In summary, we have provided the first evidence that *hub1* and *snu66* are essential eukaryotic genes that are required for pre-mRNA splicing. An important and conserved role for Hub1 is the correct subcellular localization of Snu66, although our data suggest that Hub1 is likely to perform other roles in splicing as well.

Supplemental Data

Detailed Experimental Procedures, as well as several Supplemental Figures and a Supplemental Table, are available online at http://www.current-biology.com/cgi/content/full/14/24/2283/DC1/.

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