Replicative stress induces intragenic transcription of the *ASE1* gene that negatively regulates Ase1 activity

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Summary

Intragenic transcripts initiate within the coding region of a gene, thereby producing shorter mRNAs and proteins. Although intragenic transcripts are widely expressed [1], their role in the functional regulation of genes remains largely unknown. In budding yeast, DNA replication stress activates the S-phase checkpoint that stabilizes replication forks and arrests cells in S-phase with a short spindle [2-4]. When yeast cells were treated with hydroxyurea (HU) to block DNA synthesis and induce replication stress, we found that Ase1, a conserved spindle midzone protein [5], appeared as two short protein isoforms in addition to the full-length protein. We further demonstrated that the short isoforms result from intragenic transcription of *ASE1,* which depends on the S-phase checkpoint. Blocking generation of the short isoforms leads to a destabilized S-phase spindle, characterized by increased spindle dynamics and frequent spindle collapse. Because the short Ase1 isoforms localize at the spindle in HU-treated cells and overexpression of the short Ase1 isoforms impairs the spindle midzone localization of full-length Ase1, it is likely that the presence of short Ase1 isoforms stabilizes the spindle by antagonizing full-length Ase1. Together, our results reveal intragenic transcription as a unique mechanism to down-regulate gene functions in response to DNA replication stress.

Keywords

Intragenic transcription; S-phase checkpoint; Ase1; Rad53; Spindle

Results and Discussion

The development of new technologies such as next generation RNA sequencing has allowed the identification of a large number of intergenic, intragenic, antisense and non-coding RNAs [1, 6-9]. Previous studies have described intragenic transcript isoforms where transcription initiates within the open reading frame, leading to translation from downstream

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start codons to generate truncated proteins [10-13]. However, we know little about the regulation and functional relevance of intragenic transcription. Yeast cells treated with DNA synthesis inhibitor HU arrest in S-phase with a short spindle structure [2-4], which has been shown to facilitate kinetochore-microtubule interaction [14], but cells defective in the S-phase checkpoint show elongated spindles when treated with HU. Ase1 is a spindle midzone protein that stabilizes the anaphase spindle by bundling anti-parallel microtubules [5, 15, 16]. Interestingly, overexpression of Ase1 leads to premature spindle elongation in HU-treated cells [5]. However, it is unclear how Ase1 protein function is regulated in response to DNA replication stress.

DNA replication stress induces expression of Ase1 protein fragments

Because we found that *ask1-3* kinetochore mutants show an elongated spindle after HU treatment [14], we examined if altered Ase1 protein expression is the cause. However, no obvious difference in Ase1 protein levels was detected between wild-type (WT) and *ask1-3* cells with *ASE1-13myc* after HU treatment. Surprisingly, two short protein isoforms of Ase1 appeared in both WT and *ask1-3* cells only after HU treatment (Figure 1A). The short Ase1 isoforms were also induced in synchronized cells during HU treatment, but appeared only as very faint bands during unperturbed S-phase (Figure 1B). Expression of the Ase1 short isoforms is likely specific to HU treatment because we were unable to detect them in *cdc13-1* mutant cells incubated at 34°C or in cells treated with MMS (Figure S1A and B), conditions that activate the DNA damage checkpoint [17, 18]. Therefore, HU treatment induces the expression of Ase1 short protein isoforms.

We first hypothesized that full-length Ase1 protein was being cleaved to generate the short fragments after HU treatment, but we failed to detect any Ase1 N-terminal fragments in HU-treated cells (Figure 1C). Additionally, no N-terminal protein fragments were detected in *pre9* mutants, which show reduced protein degradation [19] (Figure S1C). These results suggest that the Ase1 short protein isoforms are not cleavage products.

Intragenic transcription of *ASE1* produces a shorter mRNA isoform

To determine if shorter *ASE1* mRNAs are present during HU treatment, we performed 5' Rapid Amplification of cDNA Ends (5'-RACE), which is used to identify the sequence of the 5'-end of specific mRNAs. HU-treated cells produced much more shorter *ASE1* PCR product, while longer *ASE1* PCR products were only present in untreated cells (Figure 2A). After sequencing, we found that the short mRNA initiates between nucleotide +756 and +766 within the *ASE1* coding region (Figure 2A). To test the possibility that the full length *ASE1* mRNA is being processed to generate the short RNA, we constructed a plasmid lacking the endogenous *ASE1* promoter and the first 313 nucleotides of the coding region (*ASE1-SF*). *ase1* cells with this plasmid also express short Ase1 isoforms after HU treatment (Figure 2B). Therefore, the expression of full-length *ASE1* mRNA is not required for the production of short Ase1 protein isoforms, and *ASE1* gene likely undergoes intragenic transcription during replicative stress. We noticed an increase in Ase1 short isoforms in *ase1* cells containing *ASE1-SF* plasmid after HU treatment (Figure 2B), raising the possibility that the full-length *ASE1* mRNA may suppress the expression of the short

mRNA. However, constitutive expression of *ASE1* did not inhibit the production of Ase1 short isoforms from the *ASE1-SF* plasmid, arguing against this possibility (Figure S2A-C).

Next, we identified the start codons responsible for the short Ase1 protein isoforms. Since sequence analysis indicates that methionine residues 286 and 313 are ideal candidates, one or both of these two AUG codons were mutated to GCC (Ala) to generate plasmids with myc-tagged *ase1^{M286A}*, *ase1^{M313A}* and *ase1^{M286A/M313A}* (*ase1-AA*). Mutation of each start codon abolished the expression of one corresponding Ase1 short isoform after HU treatment, while no isoforms were detected after both start codons were mutated (Figure 2C). Thus, methionine residues 286 and 313 are the translational start sites for the short Ase1 protein isoforms.

The expression of Ase1 short isoforms is S-phase checkpoint dependent

Since the Ase1 short isoforms are only induced after HU treatment that activates the S-phase checkpoint, we asked if this induction depends on Rad53, an essential checkpoint kinase. For this purpose, we examined Ase1 protein expression in *rad53 sml1-1* checkpoint mutants, wherein the *sml1-1* mutation suppresses the lethality of *rad53* deletion by increasing dNTP production [20]. Interestingly, the Ase1 short isoforms did not appear after HU treatment in *rad53* mutant cells but were clearly induced in WT cells (Figure 3A). The expression of Ase1 short isoforms also depends on Dun1, another checkpoint kinase downstream of Rad53 [21] (Figure 3B). Together, these results indicate that the induction of Ase1 short protein isoforms requires an intact S-phase checkpoint.

We further performed northern blotting to examine the isoforms of *ASE1* mRNA using a probe specific to the 3'-region of the *ASE1* gene. In untreated cells, only the full-length *ASE1* mRNA transcript was detected. In contrast, a shorter *ASE1* transcript isoform was detected in cells treated with HU along with an obvious decrease in full-length mRNA (Figure 3C and 3D), confirming the intragenic transcription of the *ASE1* gene. In spite of the high levels of short *ASE1* mRNA in HU-treated cells, the short protein isoforms are relatively less abundant compared to full-length Ase1 protein (Figure 3A). This inconsistency could be due to a difference in protein stability. Indeed when cells were treated with HU as well as the protein synthesis inhibitor cycloheximide, full-length Ase1 remained stable while the short isoform disappeared (Figure S3A), indicating that the short isoform is less stable than the full-length protein.

We also examined the levels of *ASE1* mRNAs in *rad53 sml1-1* cells after HU treatment using northern blotting. Compared with WT cells, *rad53* mutants exhibited a reduced level of *ASE1* intragenic transcript and increased full-length *ASE1* mRNA (Figure 3C and S3B). Similar results were obtained with a *rad53-21* mutant. Rad53 has been shown to regulate replication origin firing [22], and *ARS1501* is located 229 bp upstream of the *ASE1* start codon. In a strain wherein the origin is replaced with *TRP1* (*TRP1-ASE1*), HU treatment also induced the expression of Ase1 short protein isoforms (Figure S3C), indicating that Rad53-dependent intragenic transcription of *ASE1* is not a consequence of altered transcription at the replication origin after HU treatment. Together, these data support the conclusion that intragenic transcription of *ASE1* is induced during DNA replication stress, which depends on the S-phase checkpoint.

Abolishment of the expression of Ase1 short isoforms causes HU sensitivity and altered spindle dynamics

One important question is why cells produce Ase1 short isoforms in response to HU treatment. We first examined if *ase1-AA* mutants that cannot produce the short isoforms are sensitive to HU. Indeed, *ase1-AA* cells grew slowly on plates containing 100 mM HU compared to the control cells (Figure 4A). Because *ase1Δ* mutants also show slightly slower growth on HU plates, it is possible that the mutated Ase1-AA protein loses its function. The *ase1Δ* mutant shows synthetic growth defects with other mutants such as *cin8Δ*, *kar3Δ* and *nip100Δ* [23, 24]. Our observation that the introduction of *ase1-AA* plasmid suppressed the growth defects of *ase1Δ nip100Δ* double mutants as well as the normal spindle structure in untreated *ase1-AA* mutant cells indicates that Ase1-AA protein maintains most of its function (Figure S4A). Therefore, blocking the generation of short Ase1 protein isoforms in *ase1-AA* mutants causes some defective response to replicative stress.

Because Ase1 is a microtubule-associated protein [5], the generation of the short isoforms may affect spindle morphology in HU-treated cells. Therefore, we examined the spindle structure in *ase1Δ TUB1-GFP* cells containing either *ASE1* or *ase1-AA* plasmid after G$_1$ release into HU for 3 hrs. Interestingly, we noticed a 3-fold increase in the number of cells with abnormal curved or dot-like spindles in *ase1-AA* mutants compared to control cells (the arrow in Figure 4B). We further compared spindle dynamics in those cells using live-cell microscopy. The spindle in cells with WT *ASE1* stayed relatively stable during HU arrest (Figure 4C). In contrast, the spindle in most of the *ase1-AA* cells was much more dynamic with larger variation in length. In many time points, we observed spindle collapse as evidenced by the short distance between the two spindle ends (Figure 4C, arrows). This collapse is unlikely an artifact of spindle orientation since two closely localized spindle poles were observed in the same Z stack. Introduction of *ASE1-SF* into *ase1-AA* mutant cells suppressed the abnormal spindle morphology as well as the increased spindle dynamics (Figure 4B and C). Introduction of a second copy of *ase1-AA* showed similar spindle morphology as *ase1-AA* mutant cells (data not shown), indicating that the phenotype is attributed to the absence of Ase1 short isoforms and the rescue is not simply due to higher Ase1 protein levels. Additionally, *ase1Δ* and *ASE1-SF* cells showed less dynamic spindles, although they were shorter overall (Figure S4B). Therefore, when the expression of the short Ase1 isoforms is blocked, the presence of full length Ase1 alone results in spindle destabilization during HU treatment.

The balance of pushing and pulling forces between microtubule motors regulates spindle length [25]. Ase1 recruits kinesin-5 Cin8 onto the spindle where it slides antiparallel microtubules apart [26]. Interestingly, *cin8Δ* suppressed both the HU sensitivity and the abnormal spindle structure in *ase1-AA* cells treated with HU (Figure 4A and B). *cin8Δ* also suppressed the increased spindle dynamics in *ase1-AA* (Figure 4C). These results suggest that the highly dynamic spindle morphology in *ase1-AA* mutant cells depends on the microtubule motor Cin8, at least partially. Consistently, increased Cin8 levels in HU treated cells can induce spindle elongation [27]. Thus, it is likely that Ase1-dependent Cin8 recruitment in HU-treated *ase1-AA* cells promotes the sliding of antiparallel microtubules, resulting in destabilized spindles.

The Ase1 short protein isoforms contain the microtubule-binding domain but lack the dimerization domain (Figure 2A). These isoforms likely bind microtubules but are unable to dimerize and crosslink antiparallel microtubules, thereby playing a dominant negative role by antagonizing full-length Ase1 protein. To test this idea, we first examined the growth of *cin8*, *kar3* and *nip100* mutants overexpressing the Ase1 short isoform from a galactose inducible promoter, P_{GAL}-*ASE1-SF* (Figure S4C). Previous studies show that these mutants exhibit synthetic growth defects with *ase1* [23, 24]. Interestingly, overexpression of *ASE1-SF* also caused sick growth in these mutants, supporting the dominant negative role of the short isoform (Figure S4D). We also examined the localization of endogenous full-length Ase1 in cells overexpressing *ASE1-SF*. Similar to previous studies [5, 28], we observed the restricted localization of Ase1 at the spindle midzone in control cells (Figure S4E). In 23 out of 25 cells overexpressing *ASE1-SF*, however, the localization of full-length Ase1 was dispersed and towards one end of the spindle as it elongated. Moreover, a defect in spindle elongation and spindle breakage was observed frequently in these cells (Figure S4E), similar to *ase1* mutants [5]. These data support the dominant negative role of the short Ase1 protein isoforms. Importantly, Ase1-AA-GFP shows increased localization to the spindle during HU treatment compared to WT Ase1-GFP and this increase was suppressed by introduction of the *ASE1-SF* plasmid, indicating that expression of Ase1 short isoforms reduces the spindle association of full length Ase1 (Figure 4D). Therefore, we speculate that the expression of short isoforms of Ase1 stabilizes the spindle during HU treatment by antagonizing full-length Ase1. We noticed that only a small portion of cells showed spindle localization of Ase1-GFP, which may be explained by dynamic Ase1-microtubule interaction. In *ase1-AA* cells, the association of Ase1 to the anti-parallel microtubules might be stable, but the sliding likely causes spindle collapse and the dissociation of Ase1 from the spindle.

Conclusions

Recent genome-wide isoform profiling in budding yeast identified transcription boundaries for millions of RNA molecules, revealing the presence of multiple transcript isoforms for most genes in the genome [1]. Here we provide the first evidence for S-phase checkpoint-dependent intragenic transcription in response to DNA replication stress. We found that the *ASE1* gene is subjected to intragenic transcription upon HU treatment, generating two short Ase1 protein isoforms. Moreover, intragenic transcription of *ASE1* depends on the S-phase checkpoint kinase Rad53. Importantly, our results suggest that the expression of the short Ase1 isoforms stabilizes the short spindle structure in HU-arrested cells likely by antagonizing full-length Ase1. Together, our data uncover a novel checkpoint-dependent mechanism to maintain a stable spindle structure during replication stress by inducing intragenic transcription of the *ASE1* gene. Additional genes may also undergo intragenic transcription during the cell cycle and differentiation or when cells encounter environmental challenges. Therefore, intragenic transcription is likely a unique mechanism to regulate gene function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

YPD   Yeast Peptone Dextrose

HU   Hydroxyurea

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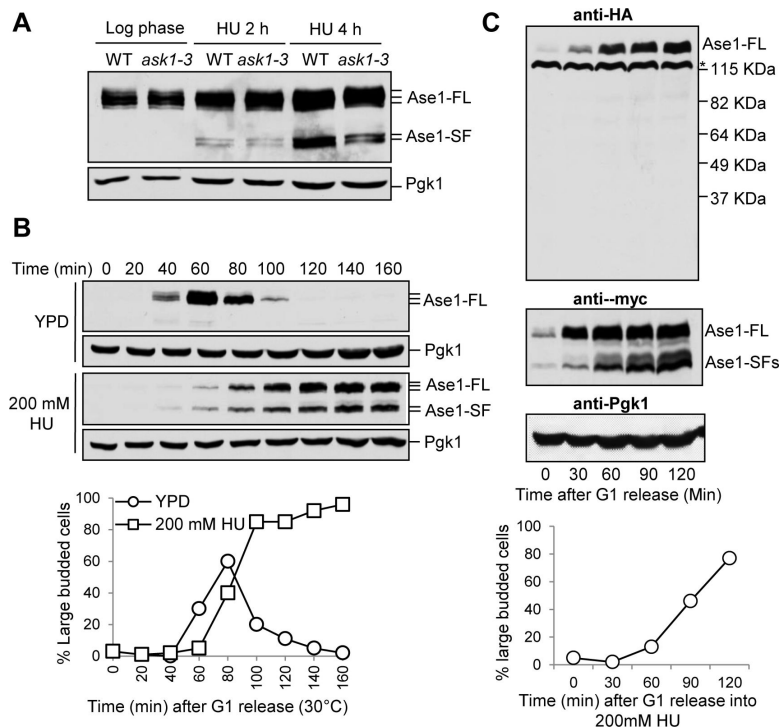
Highlights

Replicative stress induces intragenic transcription of the *ASE1* gene.

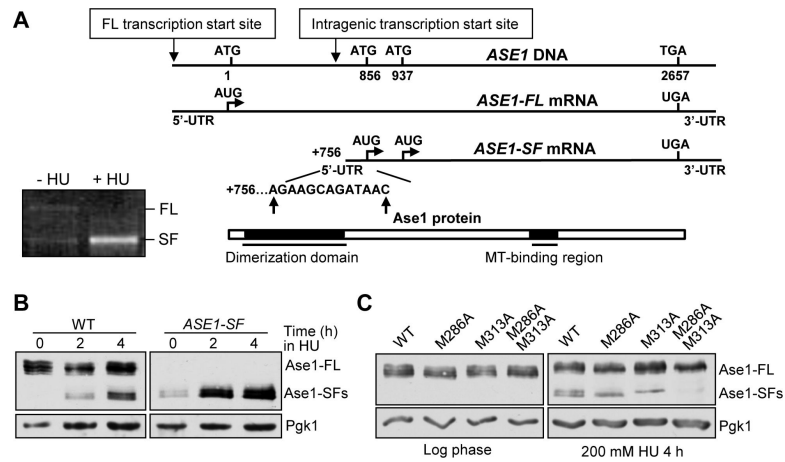The intragenic transcription of *ASE1* depends on the S-phase checkpoint.

The short Ase1 protein isoforms promote spindle stability during replicative stress.

A

Log phase | HU 2 h | HU 4 h
WT ask1-3 | WT ask1-3 | WT ask1-3

Ase1-FL
Ase1-SF

Pgk1

C

anti-HA

Ase1-FL
* 115 KDa
82 KDa
64 KDa
49 KDa
37 KDa

B

Time (min) 0 20 40 60 80 100 120 140 160

YPD

Ase1-FL
Pgk1

200 mM HU

Ase1-FL
Ase1-SF
Pgk1

anti--myc

Ase1-FL
Ase1-SFs

anti-Pgk1

0 30 60 90 120
Time after G1 release (Min)

% Large budded cells

—○— YPD
—□— 200 mM HU

Time (min) after G1 release (30°C)

% large budded cells

0 30 60 90 120
Time (min) after G1 release into 200mM HU

**Figure 1. HU-induced expression of Ase1 short protein fragments**

(A) The expression of Ase1 in HU-treated WT and *ask1-3* mutant cells. *ASE1-13myc* and *ask1-3 ASE1-13myc* cells were grown to log phase at 30°C and released to YPD containing 200 mM HU. Cells were collected at the indicated time points and protein samples were prepared for western blotting. Full length Ase1 migrates around 140 kDa and the short isoforms migrate around 105 kDa. Pgk1 protein migrates at 45 kDa and levels are shown as a loading control. **(B)** Expression of Ase1 isoforms in synchronized cells treated with HU. G_1-arrested *ASE1-13myc* cells were released into YPD or YPD containing 200 mM HU. α-factor was restored in untreated cells to block the second round of cell cycle. Cells were collected at the indicated time points to detect the expression of Ase1 protein using western blotting. Budding index was used to indicate cell cycle stage and Pgk1 protein levels are shown as a loading control. **(C)** Ase1 short isoforms are not protein cleavage products. G_1-arrested *ase1* cells containing *3HA-ASE1-13myc* plasmid were released into fresh selective media containing 200 mM HU and protein samples were prepared at the indicated time points. Western blotting with anti-HA and anti-myc antibodies was used to determine the expression of full length (Ase1-FL) and short isoforms (Ase1-SF) of Ase1 protein. Budding index was used to indicate cell cycle stage and Pgk1 protein levels are shown as a loading control. Asterisk indicates a non-specific band. Sell also Figure S1.

Figure 2. The short Ase1 protein isoforms are a consequence of intragenic transcription
(A) Transcription of *ASE1* short mRNA isoform begins at bp +756 to +766. Gel image for *ASE1* gene specific PCR products after 5-RACE. Schematic of results from 5-RACE after cloning and sequencing of PCR bands (10 colonies each). **(B)** Full-length *ASE1* mRNA is not required for expression of Ase1-SFs. *ase1* cells containing WT *ASE1* or *ASE1-SF* plasmid were grown to log phase at 30°C and released into selective media containing 200 mM HU. Cells were collected at the indicated time points and protein samples were prepared for western blotting to determine the expression of Ase1-FL and Ase1-SFs. **(C)** Translation of Ase1-SFs starts at M286 and M313. *ase1* cells containing *ASE1-13myc, ase1^{M286A}-13myc, ase1^{M313A}-13myc* or *ase1^{M286A-M313A}-13myc* plasmid were grown to log phase in selective media and released into 200 mM HU for 4 hrs. Cells were collected and protein samples were prepared for western blotting with anti-myc antibody. Pgk1 protein levels are shown as a loading control. See also Figure S2.
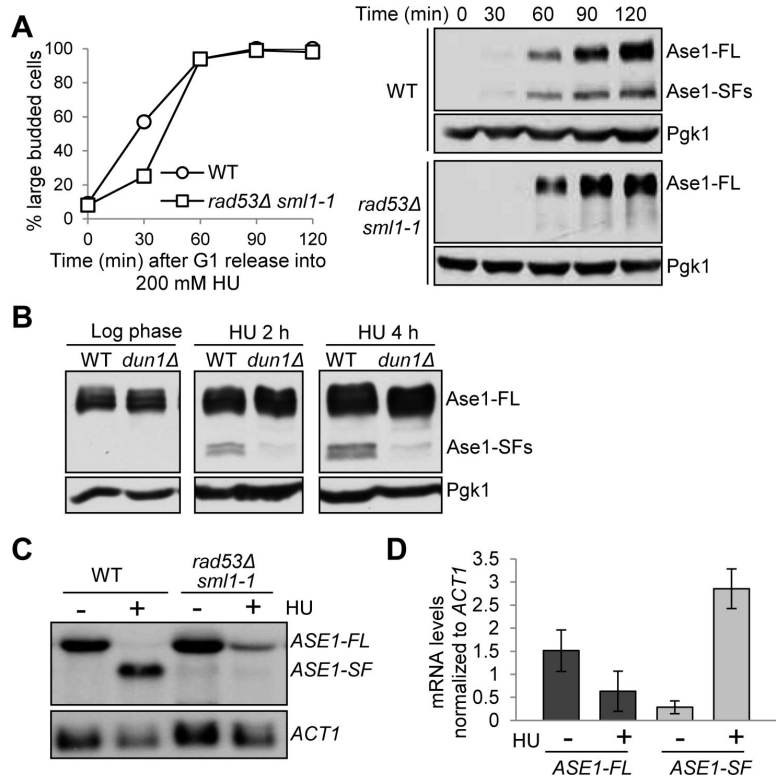
Figure 3. The expression of the Ase1 short isoforms depends on the S-phase checkpoint
(A) Rad53 is required for Ase1 short protein isoform expression. G$_1$-arrested *ASE1-13myc*
and *rad53 sml1-1 ASE1-13myc* cells were released into YPD containing 200 mM HU.
Cells were collected at the indicated time points and protein samples were prepared for
western blotting. Budding index was used to indicate cell cycle stage. **(B)** The expression of
Ase1 isoforms in *dun1* mutants. *ASE1-13myc* and *dun1 ASE1-13myc* cells were grown to
log phase at 30°C and released into YPD containing 200 mM HU. Cells were collected at
the indicated time points and protein samples were prepared for western blotting to
determine the expression of Ase1 protein. Pgk1 protein levels are shown as a loading
control. **(C)** Intragenic transcription of *ASE1* depends on Rad53. G$_1$-arrested WT and
rad53 sml1-1 cells were released to fresh YPD at 30°C. Twenty min later 200 mM HU was
added to half of the cell culture. Cells were harvested 40 min later, washed with 1xPBS and
flash frozen with liquid nitrogen. mRNA was prepared and examined by northern blotting
with a probe corresponding to nucleotides 2109 to 2411 of the *ASE1* gene. *ACT1* probe was
used for loading control. **(D)** Quantification of the *ASE1* mRNAs in WT cells treated with or
without HU from three experiments normalized to *ACT1* mRNA using ImageJ software:
ASE1-FL –HU (1.51 ± 0.45), *ASE1-FL* + HU (0.63 ± 0.43), *ASE1-SF* – HU (0.26 ± 0.14),
ASE1-SF + HU (2.86 ± 0.43). See also Figure S3.
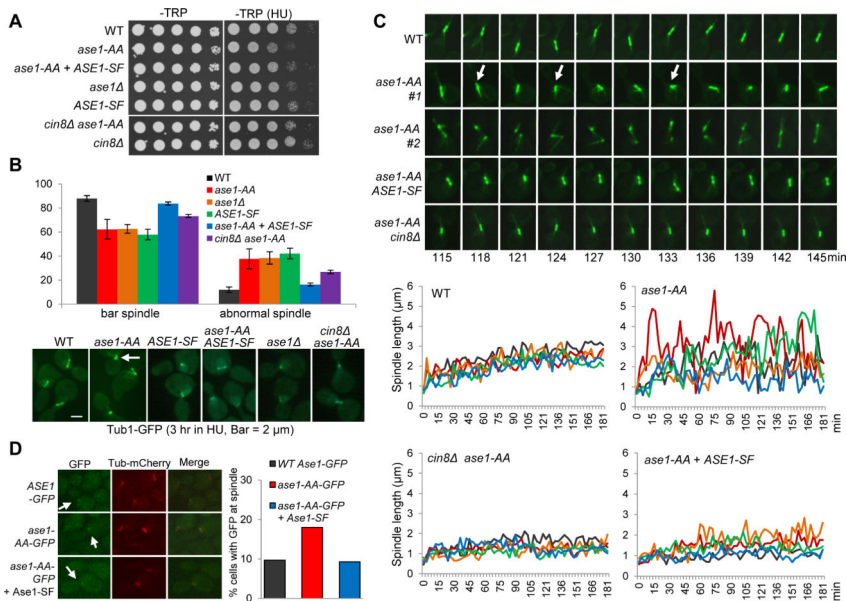
Figure 4. Expression of the Ase1 short isoforms stabilizes the S-phase spindle

(**A**) *ase1-AA* mutants are sensitive to HU. Saturated cell cultures with indicated genotypes were 10-fold serial diluted and spotted onto –TRP and –TRP + 100 mM HU plates and scanned after incubation at 30°C for 2 days. (**B**) *ase1-AA* cells show abnormal spindles after HU treatment. Cells with indicated genotypes were arrested in G_1 and released to fresh selective media containing 200 mM HU for 3 hrs at 30°C. The spindle morphology (Tub1-GFP) in representative cells is shown in the bottom panel. The white arrow indicates a collapsed spindle. The percentage of cells with a normal bar-shaped spindle and an abnormal collapsed or dot-like spindle from three biological replicates is shown in the top panel (n > 100). Normal: WT 88% ± 2.3, *ase1-AA* 62.3% ± 8.2, *ase1Δ* 62.6% ± 3.6, *ASE1-SF* 57.9% ± 4.4, *ase1-AA + ASE1-SF* 83.7% ± 1.3, *cin8Δ ase1-AA* 73.2% ± 1.5. Abnormal: WT 12% ± 2.3, *ase1-AA* 37.7% ± 8.2, *ase1Δ* 38.4% ± 5.1, *ASE1-SF* 42.1% ± 4.4, *ase1-AA + ASE1-SF* 16.3% ± 1.3, *cin8Δ ase1-A* 26.8% ± 1.5. (**C**) Spindles are more dynamic and experience more collapse in *ase1-AA* mutants during HU treatment. Thirty min following G_1 release, the cells with indicated genotypes were spotted onto the surface of a slide with an agarose medium pad containing 200 mM HU and subjected to live-cell microscopy. Five representative cells for each genotype were selected and spindle length was measured using Andor iQ2 software. Time zero was set to one image prior to the first separation of spindle poles and spindle length was plotted over time (bottom panel). Live-cell images of the spindle morphology in representative cells are shown in the top panel. Arrows indicate spindle collapse. See also Figure S4. (**D**) Ase1-SF reduces Ase1-FL spindle localization. G_1-arrested cells with indicated genotypes were released to liquid media containing 200 mM HU for 1 hr, and then mounted to a slide as described in (C). The cells were subjected to live-cell microscopy for 30 min after G_1 release for 2 hrs. The percentage of cells showing GFP localization to the spindle in several time points during the 30 min window is shown (n >100). See also Figure S4.