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A specific function for the histone chaperone NASP to fine-tune a reservoir of soluble H3-H4 in the histone supply chain

Adam JL Cook^{1,2}, Zachary A Gurard-Levin^{1,2}, Isabelle Vassias^{1,2} and Geneviève Almouzni^{1,2,†}

¹Institut Curie, Centre de Recherche, Paris, F-75248 France;

²CNRS, UMR218, Paris, F-75248 France;

[†]To whom correspondence should be addressed:

genevieve.almouzni@curie.fr, +33 (0)1 56 24 67 01

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Summary (148 words)

Proper genome packaging requires coordination of both DNA and histone metabolism. While histone gene transcription and RNA processing adequately provide for scheduled needs, how histone supply adjusts to unexpected changes in demand remains unknown. Here, we reveal that the histone chaperone Nuclear Autoantigenic Sperm Protein (NASP) protects a reservoir of soluble histones H3-H4. The importance of NASP is revealed upon histone over-load, engagement of the reservoir upon acute replication stress and when Asf1 activity is perturbed. The reservoir can be fine-tuned, increasing or decreasing depending on the level of NASP. Our data suggest that NASP does so by balancing the activity of the heat-shock proteins Hsc70 and Hsp90 to direct H3-H4 for degradation by chaperone-mediated autophagy. These insights into NASP function and the existence of a tunable reservoir in mammalian cells demonstrate that contingency is integrated into the histone supply chain to respond to unexpected changes in demand.

Introduction

The majority of cellular histones are part of chromatin, yet a small fraction – less than 1% of histone H3 and H4 in typical proliferating cells in culture (Loyola *et al.*, 2006) – are soluble. These include mainly newly-synthesized histones in transit, as well as histones evicted from chromatin during all transactions with DNA, and old or damaged histones destined for disposal (Loyola *et al.*, 2006). The normal production of histones is adapted to scheduled changes in demand through the cell cycle and at various developmental stages (Wu *et al.*, 1982; Woodland and Adamson, 1977). This is exemplified by the robust increase in histone production at the G1-S phase transition to meet the demands of chromatin replication (Wu and Bonner, 1981). Failure in proper histone supply during the cell cycle threatens genome integrity in yeast (Kim *et al.*, 1988; Prado and Aguilera, 2005; Meeks-Wagner and Hartwell, 1986) and perturbs mammalian DNA replication (Groth *et al.*, 2007). Thus, several complementary mechanisms tightly couple replication-dependent histone synthesis to DNA synthesis (Gunjan *et al.*, 2005). In mammals, this coupling is achieved by links between the cell cycle machinery, histone gene transcription and post-transcriptional regulation of histone mRNAs (reviewed in Marzluff *et al.*, 2008). Although these mechanisms are adapted to scheduled changes in demand, they are not suited to respond to sudden and unpredictable changes, raising the possibility that mechanisms which act directly on soluble histones could regulate supply to respond to sudden changes in demand.

Histone chaperones bind to histones and are involved in their transfer but are not part of chromatin (De Koning *et al.*, 2007). *In vitro*, they all promote nucleosome reconstitution (Dilworth *et al.*, 1987) yet *in vivo* each histone chaperone has a distinct function and operates in an interaction network (De Koning *et al.*, 2007). The chaperone network for histones H3 and H4, together in a hetero-dimer (Tagami *et al.*, 2004; Benson *et al.*, 2006), exemplifies this paradigm. Replicative histone variant H3.1 is deposited onto DNA coupled to DNA synthesis by Chromatin Assembly Factor 1 (CAF-1) (Smith and Stillman, 1989; Gaillard *et al.*, 1996; Tagami *et al.*, 2004). A suite of chaperones handle the replacement variant H3.3 to ensure its accumulation at distinct chromatin subdomains, including HIRA (Ray-Gallet *et al.*, 2002) and the new players DAXX and DEK (for review see Campos and Reinberg, 2010). Anti-Silencing Function 1 (Asf1) (Le *et al.*, 1997), present in both H3.1 and H3.3 complexes in human cells

(Tagami *et al.*, 2004), can donate new H3-H4 to CAF-1 (Tyler *et al.*, 1999; Mello *et al.*, 2002; Tagami *et al.*, 2004; Groth *et al.*, 2005) and likely to HIRA (Green *et al.*, 2005; Ray-Gallet *et al.*, 2007). By handling both new and old histones in coordination with the replicative helicase, Asf1 participates in the control of replication fork progression (Groth *et al.*, 2005; Groth *et al.*, 2007). A general linear scheme for H3-H4 delivery and deposition by histone chaperones has thus emerged but which chaperone actually controls the amount of soluble histones in mammalian cells is still unknown.

In this context, the presence of Nuclear Autoantigenic Sperm Protein (NASP) in H3.1 and H3.3 complexes from HeLa cells (Tagami *et al.*, 2004) drew our attention. Identified in a screen for histone binding proteins in testes (Welch *et al.*, 1990), ‘testicular’ (tNASP) and ‘somatic’ (sNASP) are generated by alternate splicing of a single gene transcript (Richardson *et al.*, 2000). Whereas sNASP is ubiquitously-expressed, tNASP is expressed highly in testes, ovaries and in transformed cells. Although considered as a linker histone chaperone (Richardson *et al.*, 2000; Alekseev *et al.*, 2003; Alekseev *et al.*, 2005; Finn *et al.*, 2008), NASP shares extensive sequence homology (O’Rand *et al.*, 1992) with the N1/N2 protein (Bonner, 1975), a histone H3-H4 chaperone in *X. laevis* (Dilworth *et al.*, 1987). Containing a canonical tetratricopeptide repeat (TPR) and 3 TPR-like domains, NASP can be structurally aligned with an expanded list of H3-H4 chaperones from fungi to mice which together form the Sim3-Hif1-NASP interrupted TPR (SHNi-TRP) super-family (Dunleavy *et al.*, 2007). Further, NASP has H3-H4 chaperone activity *in vitro* (Wang *et al.*, 2008; Osakabe *et al.*, 2010) and its homologues including Hif1p (*S. cerevisiae*) (Ai and Parthun, 2004) and Sim3 (*S. pombe*) (Dunleavy *et al.*, 2007) have *in vivo* H3-H4 chaperone activity. Furthermore, the association of N1/N2 with the stored maternal H3-H4 pool in *X. laevis* oocytes (Kleinschmidt *et al.*, 1985) provides support to the hypothesis that NASP may be key for storing soluble H3-H4. Notably, upon acute replication stress in human cells, new soluble H3-H4 that cannot be incorporated are buffered in a multi-chaperone complex containing sNASP, Asf1 and RbAp48 (Groth *et al.*, 2005). However which chaperones in this complex are key for enabling histone accumulation remains unknown. In yeast, a Rad53- and proteasome-dependent pathway degrades histones that are in excess of cellular requirements (Gunjan and Verreault, 2003; Singh *et al.*, 2009). Histone degradation, poorly understood in mammals, likely needs to be balanced to allow the accumulation of soluble histones. Which factors simultaneously keep histone degradation in check and

dynamically modulate histone supply at the protein level have not yet been identified. We hypothesized that NASP, by protecting soluble H3-H4 against degradation, could have a specialized function in human cells to fine-tune a soluble reservoir of H3-H4.

To test our hypothesis, we manipulated the level of NASP in human cell lines and assessed the effect on the soluble histone pool. Our data reveal a role for NASP in the histone chaperone network to maintain a reservoir of H3-H4. Modulation of the reservoir has direct consequences for the cell in dealing with H3-H4 over-load, buffering during acute replication stress and compromised Asf1 function. Furthermore, our data suggest that NASP protects soluble H3-H4 from degradation by chaperone-mediated autophagy, opening new avenues for understanding how histone metabolism is regulated.

Results

Fine-tuning of a soluble pool of H3-H4 by NASP

To analyze soluble pools of histone H3-H4 and associated proteins we used a well-characterized fractionation method (Figure 1A, S1A) (Loyola *et al.*, 2006). We tested how NASP can impact upon soluble histones by depleting tNASP and sNASP from cells by transfection with siRNAs (Figure S1B). Strikingly, we observed that NASP depletion substantially decreased the level of soluble H3 and H4 (Figure 1B), consistent with a recent report (Campos *et al.*, 2010), without their level being affected in the salt-extractable extract (Figure 1B). We observed this effect with multiple NASP siRNAs (Figure 1B) and cell lines (U-2 OS, HeLa or 293) (Figures 1B, 1C, 1D & S1C). This was specific for H3-H4, since we did not observe any alteration in the level of soluble H2A (Figure S1D) or H2AZ (Figure 1D), nor that of histone H1 in the salt-extractable extract (Figure 1B). To carefully quantify soluble H3 levels, we digitally acquired signals for H3 and a loading control (Figure 1B and S1E) and determined that the average decrease in soluble H3 was about 10-fold, down to as little as 12% than in control extracts (Table S1). We also examined cell proliferation, cell cycle profile or S phase progression after NASP depletion and did not detect obvious effects (Figure S1 G, H, I).

We previously identified NASP in e-H3.1 and e-H3.3 complexes, but not e-CENP-A complexes, using cell lines expressing epitope-tagged H3 variants (Tagami *et al.*, 2004). We next took advantage of these cell lines, in which the transgenes are not under the control of an endogenous histone gene promoter and do not encode for mRNAs containing known histone post-transcriptional regulatory sequences, to assess if tagged variants are affected by NASP depletion. We found that soluble e-H3.1 and e-H3.3, but not e-CENP-A, were both reduced by NASP depletion (Figure 1C) without affecting their levels in the salt-extractable fraction (Figure S1C). We further verified by quantitative RT-PCR analysis that NASP depletion did not reduce H3 or H4 RNA levels (Figure S1F). Thus, we conclude that NASP depletion specifically affects H3-H4 soluble pools without affecting their transcripts, possibly by stabilizing soluble H3-H4 proteins following their synthesis.

To formally demonstrate that it is indeed NASP itself that maintains soluble H3-H4 levels, we established 293 Flp-In cell lines stably expressing e-NASP isoforms rendered insensitive to siNASP #1.

By rescuing NASP expression in this way we could confirm the specificity of the effect following siRNA treatment. Transfection of these cells with siNASP #1 depleted endogenous NASP, leaving principally e-tNASP or e-sNASP (Figure 1D). Contrary to control cells (Figure 1B, 1C), transfection of e-sNASP or e-tNASP-expressing cells with siNASP did not appreciably affect soluble H3 and H4 levels, indicating that both NASP isoforms function to maintain the reservoir of soluble H3-H4 *in vivo* (Figure 1D).

We wondered whether NASP functions to maintain a pool of H3-H4 that can expand or contract according to NASP availability. To test if NASP over-expression increases soluble H3-H4 levels, we established 293 Flp-In cell lines in which either sNASP or tNASP can be over-expressed from a tetracycline-inducible transgene. We found that over-expression of either isoform was paralleled by an increase in the level of soluble H3 and H4 (Figure 2). NASP over-expression did not affect the cell cycle (Figure S2A), non-nucleosomal CENP-A levels or the level of salt-extractable H1 (Figure S2B), confirming the specificity for modulation of H3.1/H3.3 and H4 levels. Thus altering the level of NASP can modulate a reservoir of soluble H3-H4 in either direction.

NASP is unique amongst its partners to fine-tune a soluble pool of H3-H4

To determine whether NASP is unique in its ability to maintain the soluble pool of H3-H4, we depleted partners which are retrieved when pulling down NASP (Figure 3A). Depletion of Asf1 did not alter the level of soluble histone H3 (Figure 3B). This distinct role for NASP is consistent with the fact that NASP and Asf1 can bind independently to H3-H4 (Figure S3). We next wondered whether the loss of soluble histones was due to impaired acetylation of H4 by HAT1, another protein in complex with NASP (Figure 3A). We found that in contrast to NASP depletion, HAT1 depletion and the resultant reduction in H4 acetylation, did not affect soluble H3-H4 levels (Figure 3C). Thus NASP is able to stabilize soluble H3-H4 independently of any role it may have in facilitating H4 acetylation.

We next sought to identify the region of H3 that mediates its direct interaction with NASP. A hint came from the observation that *in vitro*, sNASP less efficiently assembled H3.1 with a V71M point mutation in its N-terminus into nucleosomes (Osakabe *et al.*, 2010). *In vitro* pull-down assays using fragments of recombinant H3 showed that, unlike His₆-Asf1a, His₆-NASP bound to a fragment encompassing amino acids 1 to 79 of H3 (Figure 3D). In contrast, His₆-Asf1a, but not His₆-NASP, bound to amino acids 85 to the C-terminus of H3, the region with which Asf1 is known to interact in the context

of the H3-H4 dimer (Mousson *et al.*, 2005). We thus propose that *in vivo*, NASP is able to interact directly with the H3-H4 dimer *via* the N-terminal region of H3.

NASP affects the cellular response to perturbed histone supply

While the NASP-dependent H3-H4 reservoir is dispensable under normal proliferation conditions, we wondered whether it could be important when the pool of free histones is altered. We tested this possibility by artificially creating an excess of new H3-H4 in NASP-depleted U-2 OS cells by transfecting them with a plasmid to co-express e-H3.1 and H4-EGFP (Figure 4A), a condition that impairs S phase progression due to disruption of normal Asf1 activity (Groth *et al.*, 2007). We analyzed the cell cycle distribution of cells over-expressing H3-H4 and found that NASP depletion partially alleviated their accumulation in S phase. As for endogenous H3 and stably-expressed e-H3 (Figure 1B, 1C), NASP depletion reduced the level of soluble e-H3.1 expressed by transient transfection (Figure S4A). We next sought to test if NASP over-expression, which increases the soluble pool of H3-H4 (Figure 2), could likewise impair the cellular response to excess new H3-H4. In contrast to NASP depletion, over-expression of EGFP-tagged sNASP exacerbated the accumulation in S phase of cells specifically with high H3-H4 over-load (Figure 4B, S4B). The same trend was observed for cells co-transfected with a plasmid from which the ectopically-expressed sNASP is untagged or one which allows tracking of ectopic sNASP expression by virtue of an sNASP-IRES-EGFP cassette (not shown). These findings show that modulation of the soluble pool of H3-H4 by NASP can have tangible consequences for histone supply, particularly when another point in the histone supply chain is perturbed.

Asf1 has been implicated as a donor of new histones H3-H4 within the histone supply chain. One prediction is that loss of the NASP-dependent H3-H4 reservoir, while not affecting proliferation under normal conditions, could aggravate S phase progression when Asf1 function is directly perturbed. We thus transfected U-2 OS cells with siRNAs against NASP and Asf1 and analyzed their cell cycle. Whereas NASP depletion alone did not affect the cell cycle, it had a pronounced effect on Asf1-depleted cells (Figure 4C, *middle*). This effect was even more pronounced when we attempted to synchronize the cells with a double thymidine block (Figure 4C, *right*). Western blotting indicated that this was not due to a reduction in soluble H3 level beyond that due to NASP depletion (Figure S4C). Interestingly, NASP

depletion alone considerably reduced the level of soluble Asf1, but had only a minor effect on the Asf1 level in siAsf1-transfected cells. As Asf1 depletion did not affect the level of soluble H3-H4 (Figure 3B), NASP has a dominant role in the control of soluble H3-H4 levels.

Fine-tuning of the soluble pool of H3-H4 upon rapid change in demand during replication stress

A sudden halt to DNA replication upon treatment with HU causes newly synthesized histones to back-up (accumulate) in a multi-chaperone complex containing NASP (Groth *et al.*, 2005). To investigate whether their accumulation requires NASP, we depleted NASP in U-2 OS cells, synchronized them in S phase and treated them with HU (Figure 5). We confirmed by flow cytometry that both siControl- and siNASP-transfected cells were in mid-S phase (Figure S5A). We analyzed extracts prepared from S phase cells with ('HU') or without ('S') treatment and observed that, as anticipated, treatment of siControl-transfected S phase cells with HU lead to a subtle but reproducible ~2-fold increase in the level of soluble H3 (Figure 5). The level of H3 in soluble extracts from siNASP-transfected cells either remained steady or increased only slightly as a result of HU treatment. Careful quantification of the difference in H3 levels (as in Figure S1E) confirmed that the HU versus S phase soluble H3 ratio was always lower from siNASP- than siControl-transfected cells (Figure 5 and Figure S5). NASP is thus required to stabilize H3-H4 that suddenly cannot be incorporated during replication stress.

NASP protects soluble H3-H4 from degradation by chaperone-mediated autophagy

In *S. cerevisiae*, excess soluble histones H3-H4 are degraded by a ubiquitin-proteasome pathway (Gunjan and Verreault, 2003; Singh *et al.*, 2009). To test whether NASP protects histones H3-H4 against degradation, we tested the impact of several different chemical proteasome inhibitors on soluble histones H3-H4 levels. Although proteasome inhibition reproducibly lead to a small increase in the level of soluble H3 and H4 in siControl- and siNASP-transfected cells, it never restored the levels in siNASP-transfected cells to that in siControl-transfected cells (Figure 6B). The levels of endogenous and epitope-tagged H3 and H4 expressed by transient or stable transfection were similarly affected by proteasome inhibition (not

shown), using different chemical inhibitors (not shown) on both HeLa S3 (Figure 6) and U-2 OS cells (not shown).

We next considered whether chaperone-mediated autophagy (CMA), an alternative degradation pathway by which client proteins are targeted by heat-shock protein Hsc70 and its co-chaperones for lysosomal destruction (Figure 6A)(Cuervo, 2010), could be involved. Indeed Hsc70 and Hsp90 are associated with soluble H3-H4 (Campos *et al.*, 2010; Alvarez *et al.*, 2011). Depletion of Hsc70 or Hsp90 β in HeLa S3 cells using siRNAs lead to a striking increase in the levels of soluble H3-H4 (Figure 6C and S6A) and H2A (data not shown). The effect was observed with multiple siRNAs for each target (not shown). Further, the depletion of Hsp90 α , the inducible isoform, did not increase soluble histone levels (not shown). Importantly, the level of soluble H3-H4 in cells co-transfected with siHsc70 or siHsp90 β and siNASP was able to reach that in cells transfected with siHsc70 or siHsp90 β alone (Figure 6C). This contrasted with proteasome inhibition, which did not rescue soluble H3-H4 levels in NASP-depleted cells (Figure 6B).

To confirm that the increased histone levels are due to impairing their degradation *via* CMA, we transfected cells with a siRNA targeting Lamp-2a, the receptor for Hsc70 on the lysosomal surface. Lamp-2a depletion increased soluble H3-H4 levels to the same extent in control and NASP-depleted cells (Figure 6D and S6B). This was not due to a non-specific increase in soluble protein levels because the levels of several other soluble proteins including NASP, Asf1 and CENP-A were not appreciably altered by Hsc70, Hsp90 β or Lamp-2a depletion (Figures 6C, 6D, S6A and S6B). Since we found that the levels of H3, H4 and H1 in whole-cell extracts were not increased by Lamp-2a depletion (Figure S6B) and the majority of cellular histones are part of chromatin, we conclude that defective CMA causes an accumulation specifically of non-nucleosomal histones.

The depletion of Hsc70, Hsp90 β or Lamp-2a not only rescued the loss of soluble H3-H4 in NASP-depleted cells but also increased the levels beyond that reached by NASP over-expression (Figure 2) suggestive of evicted histone accumulation. By analyzing post-translational modifications of histones in soluble extracts from Lamp-2a-depleted cells, we found that in addition to H4K12ac, a mark corresponding to newly-synthesized H4, H3K9me3, H3K27me3 and H3K49me1, modifications enriched on nucleosomal H3, also increased (Figure 6D). This accumulation was unlikely a result of non-specific

release of proteins from chromatin since HP1 α , a protein enriched on chromatin, did not appreciably accumulate in the soluble fraction (Figure S6A & 6D). We conclude that disruption of CMA impedes the degradation of new and evicted histones.

Discussion

A reservoir of H3-H4 histones.

New histones are thought to enter a “just-in-time” linear supply chain in which histone production is coupled to the immediate demands for chromatin assembly. Here we report that in somatic cells, NASP maintains H3-H4 in a reservoir (Figure 1) that is dispensable during normal proliferative conditions (Figure S1). Conceptually, it implies that contingency can be integrated into the supply chain to cope with unanticipated changes in demand or impaired histone production (Figure 7). Indeed, the reservoir affects the cellular response to both artificial H3-H4 over-load (Figure 4A,B) and perturbation of Asf1 function, impinging on S phase progression (Figure 4C). This function of NASP in dealing with the H3-H4 reservoir is unique, and distinct from the function of Asf1 in handling new H3-H4 (Figure 7). This is further emphasized by their binding to different domains of H3 (Figure 3D and Mousson *et al.*, 2005) and the different outcomes when they are depleted.

We have shown that NASP is required to stabilize newly-synthesized H3-H4 upon an unexpected block to replication (Figure 5). This storage pool could ensure short-term provision of histones until normal histone production resumes. In cells experiencing high levels of replication stress and thus fluctuating histone synthesis, the NASP-dependent reservoir may expand, providing contingency to ensure S phase progression. This is reminiscent of the firing of dormant replication origins to ensure proper genome duplication during replication stress (recently reviewed in (Blow *et al.*, 2011)). The over-expression of NASP observed in several cancer cell lines (Richardson *et al.*, 2001) and ovarian cancer (Ali-Fehmi *et al.*, 2010) may thus either represent (i) a means to cope with replication stress, a hallmark of cancer (Negrini *et al.*, 2010) known to lead to genomic instability (Halazonetis *et al.*, 2008), or; (ii) directly contribute to replication stress if its over-expression leads to an over-supply of H3-H4. Conversely, reducing the level of NASP compromises the H3-H4 reservoir and this could also threaten genome stability although by other means. Indeed, improper histone supply in *S. cerevisiae* has been linked to replication fork stalling (Prado and Aguilera, 2005), gene amplification (Libuda and Winston, 2010) and unfaithful chromosome transmission through mitosis (Meeks-Wagner and Hartwell, 1986). It

will be interesting to determine how compromised NASP function, either over- or under-expression, can threaten genome integrity and play a role in tumorigenesis.

The important role of NASP is underscored by the fact that it is essential for early mouse embryonic development (Richardson *et al.*, 2006). It is tempting to speculate that NASP is actually crucial to maintain a reservoir of H3-H4 required for the dynamic chromatin reorganization that occurs following fertilization. Further situations during development can similarly require extra histone supply. In this regard, it is interesting to note that NASP expression is high in embryonic stem (ES) cells and decreases upon differentiation (Sun *et al.*, 2008; Yocum *et al.*, 2008). Finally, since chromatin-bound histone H3 and H4 levels are reduced in aged human cells (O'Sullivan *et al.*, 2010) and artificially increasing histone synthesis can counteract aging of *S. cerevisiae* (Feser *et al.*, 2010), NASP could represent an interesting target to consider to prevent the cellular aging process

Fine-tuning the H3-H4 reservoir: a balance between NASP and chaperone-mediated autophagy

Fine-tuning of this reservoir by NASP (Figure 1) provides a novel means to control histone availability. Our results show that in somatic mammalian cells this reservoir can be expanded up to double its normal size, or shrunk it to as little as 10%, as the level of NASP is varied (Figures 1, 2). In oocytes of various species, the soluble pool can increase up to a hundred times greater than the histone component in chromatin (Woodland and Adamson, 1977; Laskey *et al.*, 1977; Wassarman and Mrozak, 1981) and in *Xenopus* the NASP homologue N1/N2 has been implicated in this storage.

In *S. cerevisiae*, a Rad53- and proteasome-dependent pathway is required for degradation of excess soluble histones (Gunjan and Verreault, 2003; Singh *et al.*, 2009). We observed that treatment of the human cells used in our study with proteasome inhibitors had only a subtle effect on soluble histone levels (Figure 6B). Our results instead point toward a new role for chaperone-mediated autophagy (CMA) in degrading histones (Figure 6 and S6). We propose that in mammalian cells the H3-H4 reservoir is fine-tuned by a dynamic balance between degradation by CMA and protection by NASP (Figure 7). Although Hsc70 and Hsp90 may initially aid with histone folding and stabilization (Campos *et al.*, 2010), they could switch function (Höhfeld *et al.*, 2001; Young *et al.*, 2004) dispatching histones for degradation if

the new histones are misfolded or cannot be handed over to downstream chaperones. Further, whether histones evicted from chromatin are also handled by the heat-shock proteins and targeted for CMA will be of particular interest due to the potential insight into how the landscape of histone PTMs on chromatin is regulated. We have described here a tunable reservoir of H3-H4 controlled by NASP, however analogous tunable systems should be considered for other histones and examined in different cell types. While we provide an important insight into the role of CMA in degrading soluble histones, characterizing the molecular details of this pathway in the regulation of histone supply will be an avenue to explore in the future.

Experimental Procedures

Antibodies.

For NASP anti-serum, human tNASP-His₆ fusion protein was expressed in *E. Coli*, purified on Nickel-NTA His-bind resin (Novagen), and then injected into rabbits (AgroBio, France). Rabbit polyclonal anti-sera against Asf1a & b, were previously described (Groth *et al.*, 2005; Green and Almouzni, 2003). H4K5,12ac anti-serum was raised by injecting rabbits with a histone H4 peptide (SGRGK₅acGGKGLGK₁₂acGGAKRHR) diacetylated on lysines 5 & 12 (Proteogenix). Dot blotting confirmed that the anti-serum recognizes K12ac or K5,12ac peptides, K5ac (weakly), but not unmodified peptide (not shown). Other antibodies were: histone H3 (Abcam ab1791), histone H4 (clone 62-141-13, Upstate 05-858), CENP-A (clone 3-19, Abcam, ab13939), histone H1 (Abcam ab1938), histone H2AZ (Millipore 09-862), H4K12ac (Abcam ab1761), H3K9me3 (Upstate 07-442 lot:DAM1411287), H3K27me3 (Upstate 07-449 lot:DAM1514011), H3K79me1 (Abcam ab2886 lot:527479), HAT1 (Abcam ab12164), CAF-1 RbAp48 (Abcam ab1765), GAPDH (Abcam ab9485) HP1 α (Sigma H2164), Lamp-2a (Abcam ab18528), γ -tubulin (clone GTU-88, Sigma T5326), Hsc70 (Abcam ab19136), Hsp90 β (clone K3705, Enzo ADI-SPA-842), β -catenin (BD Signal Transduction, 610153), StrepMAB-Classic (IBA, 2-1507-001), HA epitope tag (clone 3F10, Roche 1867423)

Plasmids and cell lines.

tNASP and sNASP cDNAs were amplified by PCR from a commercial clone (Open Biosystems #3538429) and from 293 cell cDNA, respectively, and ligated into pGEX-4T-1 (GE Healthcare) and pET-30a(+) (Novagen) plasmids. Sequences were confirmed to be correct by sequencing and comparison to Genbank accessions NM_002482.2 (tNASP) and NM_152298.2 (sNASP). NASP cDNA was subcloned into pEGFP-N1 or pEGFP-C1 (Clontech) for creating NASP-EGFP expression plasmids and verified by sequencing. In order to express NASP with a C terminal OneSTrEP tag (WSHPQFEKGGGSGGGSGGGWSHPQFEK) separated by a minimal linker (GASS), a dsDNA adapter was ligated immediately 3' of sNASP or tNASP in pNASP-EGFP. The resultant NASP-OneSTrEP (e-NASP) sequences were transferred to pcDNA5/FRT (Invitrogen) and these plasmids stably transfected into 293 Flp-In cells by selection with 150 μ g/mL Hygromycin. For tetracycline-inducible

NASP expression, NASP cDNA was transferred from pGEX-4T-1 to pcDNA5/FRT/TO (Invitrogen) and the plasmids stably transfected into 293 Flp-In T-Rex cells. To establish 293 Flp-In cell lines stably-expressing e-NASP resistant to siNASP #1, site-directed PCR mutagenesis was performed on e-NASP plasmids using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) to introduce three point mutations (G954A, G957A and G960A corresponding to the sNASP cDNA sequence). The plasmid for co-expression of e-H3.1 (H3.1-Flag-HA) and H4-EGFP (pBI-eH3.1-H4-EGFP) was previously described (Groth *et al.*, 2007). EGFP was swapped for mCherry by restriction digest and ligation to create the variant pBI-eH3.1-H4-mCherry. Transfections with these plasmids were performed with U-2 OS cells stably transfected with pTet-Off (Clontech).

***In vitro* interaction studies**

NASP cDNAs were cloned into pET-30a(+) plasmid for expression of His₆-NASP in *E. Coli*. Asf1a cDNA was sub-cloned from GST-Asf1a plasmid (as previously described Groth *et al.*, 2005) into pET-30a(+) for expression of His₆-Asf1a. For the production of full-length and truncated H3.2 with N-terminal GST tag, *Drosophila* histone H3.2 cDNAs (*Drosophila* H3.2 amino acid sequence is identical to human H3.2) were cloned by PCR into pGEX-4T-1 and proteins expressed in *E. Coli*. GST-tagged proteins were linked to glutathione Sepharose 4B (GE Healthcare) then incubated with purified His₆-tagged proteins in binding buffer (20 mM Tris HCl pH 7.4, 600 mM NaCl, 1 mM MgCl₂, 1 % (v/v) NP-40, 0.5 mM DTT, 0.1% (v/v) Tween 20 and Complete protease inhibitor (no EDTA) (Roche) for 2 hours at 4°C. After washing with binding buffer, bound proteins were eluted directly in SDS-PAGE loading buffer.

Cell culture and transfections.

U-2 OS cells were provided by J Bartek; e-H3.1/H3.3/CENP-A HeLa S3 cell lines were previously described (Tagami *et al.*, 2004; Dunleavy *et al.*, 2009); 293 Flp-In and Flp-In T-REx cells were from Invitrogen. Cells were cultured in DMEM (Gibco) supplemented with 10% fetal calf serum (Eurobio). SiRNA transfections were performed using Lipofectamine RNAiMAX (Invitrogen) and 50 nM siRNA final concentration. Plasmids transfections were performed using Lipofectamine 2000.

Cellular protein fractionation.

Extracts were prepared essentially as previously described (Martini *et al.*, 1998). Briefly, adherent cells were swollen in hypotonic buffer (20 mM HEPES.KOH pH 7.8, 5 mM Potassium acetate, 0.5 mM MgCl₂, & protease inhibitors), collected with a cell scraper & disrupted with 25 strokes of a Dounce homogenizer. Following centrifugation (1500g), the supernatant (soluble extract) was reserved and the pelleted nuclei were incubated for 1.5 h with gentle mixing in buffer (as above) containing 600 mM NaCl and 10% glycerol. Centrifugation at 14,000 rpm (18,188g) in a benchtop centrifuge (Eppendorf) for 20 min was performed to obtain a salt-extractable fraction (the supernatant) and an insoluble pellet. See Fig S1A for detailed explanation and references.

Gel electrophoresis, Western blotting and quantification.

We used the NuPAGE® Electrophoresis System with Novex Bis-Tris pre-cast 4-12% gradient gels and MES buffer (Invitrogen) & 0.2 µm Protran® nitrocellulose membrane (Whatman) with the Trans-Blot SD semi-dry transfer cell (Bio-Rad). Primary antibody labeling was revealed with HRP-conjugated secondary antibodies (Jackson ImmunoResearch), SuperSignal West Pico or Femto chemiluminescent substrate (Pierce) and exposure to film. For quantification, signals were captured on the Chemidoc system (Bio-Rad) & QuantityOne software (Bio-Rad) was used to determine signal intensities using the volume analysis tool. Signal intensities were plotted against sample input for the dilution series using Excel software (Microsoft) and gradients for determined for lines of best linear fit for each dilution series. The relative H3 levels between two samples were calculated as the ratio of their gradients.

siRNAs.

Sense strand (5'-3') sequences as ordered from Dharmacon: Control (ON-TARGETplus non-targeting siRNA #1, D-001810-01); NASP #1 and #2 designed by Dharmacon (using Genbank NM_152298), ON-TARGETplus J-011740-16 (GUACUAAACGAGCAGGUGA) & J-011740-13 (GGAACUGCUACCCGAAAUU); NASP #3 (GCACAGUUCAGCAAAUCUAdTdT) (Richardson *et al.*, 2006), and; Asf1 siRNAs a2 and b1 as described (Groth *et al.*, 2005). From Eurofins MWG Operon: Luciferase (CGUACGCGGAUACUUCGA), Hsc70 (HSPA8 (NM_006597.3),

CCUAAAUUCGUAGCAAAUdTdT),	Hsp90 β	(HSP90AB	(NM_007355.2),
AGAGGAGUAUGGAGAAUUCdTdT),		Lamp-2a	(NM_002294,
AGACUGCAGUGCAGAUGACdTdT)	&	HAT1	(NM_003642.3,
GCUACAGACUGGAUAUUAAdTdT).			

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Figure Legends

Figure 1. NASP maintains a soluble pool of histones H3.1/H3.3-H4.

A. Experimental and cellular fractionation scheme for assessing the effect of NASP depletion on soluble and salt-extractable extracts. See Figure S1A and Experimental Procedures for full details.

B. siRNA-mediated depletion of NASP destabilizes the soluble – but not salt-extractable – pool of histones H3 and H4. Soluble (*left*) and salt-extractable (*middle*) extracts from U-2 OS cells transfected with one of three different NASP siRNAs or Control siRNA were analyzed by SDS-PAGE and Western blotting with NASP anti-serum and specific histone antibodies. For all panels, a 2x dilution series of each extract was loaded. Memcode staining of total proteins was performed to confirm equal sample loading. *Right*, Representative digitally acquired H3 and γ -tubulin signals for soluble extracts from NASP-depleted and control cells analyzed as in B. Quantification was performed according to Figure S1E and Experimental Procedures to calculate the H3 level in NASP-depleted extracts (11.5%) relative to control (set at 100%).

C. NASP depletion leads to a loss of soluble H3.1 and H3.3. Soluble extracts from siRNA-transfected HeLa S3 cell lines stably expressing epitope-tagged H3.1, H3.3 or CENP-A, were analyzed as in B. Endogenous H3 variants were detected with H3 (recognizing H3.1 and H3.3) or CENP-A antibodies; Epitope-tagged H3/CENP-A were detected with anti-HA antibody. See also Figure S1C.

D. Either e-tNASP or e-sNASP can maintain the soluble H3-H4 pool upon endogenous NASP depletion. *Top*, Experimental scheme. We created 293 Flp-In cell lines expressing epitope-tagged tNASP or sNASP transgenes (e-tNASP and e-sNASP) resistant to downregulation by siNASP #1. Cells were transfected with siNASP #1 or siControl and, *bottom*, a 2x dilution series of soluble extracts were analyzed by SDS-PAGE and Western blotting with specific antibodies. Histone H3 and H4 levels in salt-extractable fractions were not affected by NASP depletion (not shown).

Figure 2. NASP over-expression increases the soluble H3-H4 pool.

NASP over-expression increases the level of soluble H3-H4. *Top*, Experimental scheme. We created 293 Flp-In cell lines stably carrying tetracycline-inducible tNASP or sNASP transgenes. Soluble extracts were prepared from cells treated for 48 h or not with tetracycline. *Bottom*, a 2x dilution series was analyzed as in Figure 1B.

Figure 3. NASP interacts with the N-terminus of H3 and has a unique function to maintain a soluble pool of H3-H4.

A. NASP is in complex with multiple partners *in vivo*. *Top*, H3, H4 and Asf1 co-immunoprecipitate with NASP. U-2 OS soluble and salt-extractable extracts were prepared as in Figure 1A and proteins were immunoprecipitated with either NASP or Control (pre-immune) anti-serum and analyzed by SDS-PAGE and Western blotting with specific antibodies. Input, 10%. *Bottom*, Both NASP isoforms are in multi-chaperone complexes with H3-H4 *in vivo*. Soluble extracts were prepared from 293 Flp-In cells stably expressing OneSTrEP-tagged sNASP or tNASP (e-sNASP, e-tNASP) or control (empty vector) transgenes and NASP complexes were isolated by STrEP-Tactin affinity pull-down. e-NASP complexes were eluted from the resin and analyzed by Western blotting with specific antibodies. Input, 5%.

B. siRNA-mediated depletion of Asf1 does not destabilize the soluble pool of H3-H4. Soluble extracts from U-2 OS cells transfected with Asf1a+b or Control siRNAs, analyzed as in Figure 1B. A 2x dilution series was loaded and Memcode staining was performed to confirm equal sample loading.

C. HAT1 depletion and the ensuing reduction in H4 acetylation does not destabilize the soluble pool of H3-H4. Soluble extracts from HeLa S3 cells transfected with NASP, HAT1 or Control siRNAs, analyzed as in Figure 1B. Two dilutions (1x, 3x) of each extract were loaded.

D. NASP binds preferentially to an N-terminal domain of histone H3. *Left*, Scheme showing the recombinant GST-histone H3.2 proteins used in this study. Grey boxes indicate histone fold domains. *Right*, GST-H3.2 and unfused GST proteins bound to Glutathione sepharose were incubated with recombinant His₆-tagged sNASP, tNASP or Asf1a. Bound proteins were detected by SDS-PAGE and Western blotting with specific antibodies. Input, 5%.

Figure 4. NASP affects the cellular response to perturbed histone supply.

A. NASP depletion alleviates the S phase defect resulting from H3-H4 over-supply. *Left*, Experimental scheme. NASP or Control siRNA-transfected U-2 OS cells were transfected with plasmids from which either EGFP or eH3.1+H4-EGFP is expressed. Cells were collected for flow cytometry 28 h following plasmid transfection. *Right*, Flow cytometry analysis of the cell cycle (DAPI staining) of cells gated on high EGFP fluorescence (high excess of e-H3.1+H4-EGFP or control EGFP-positive cells), transfected with either NASP or Control siRNAs.

B. sNASP over-expression aggravates the S phase defect resulting from H3-H4 over-supply. *Left*, Experimental scheme. U-2 OS cells were co-transfected with a plasmid to co-express e-H3.1 and H4-mCherry or EGFP alone (control) and a plasmid to express sNASP fused to EGFP (at the C or N terminal, sNASP-EGFP or EGFP-sNASP, respectively). Thirty hours following transfection the cells were harvested for flow cytometry. *Right*, Flow cytometry analysis of the cell cycle profiles of cells gated for high EGFP expression (high expression of sNASP-EGFP fusion protein or EGFP control) and high mCherry intensity (high excess of e-H3.1+H4-mCherry). Note that in contrast to Fig 4A, the cells were fixed with paraformaldehyde (to retain the GFP fusion proteins), prior to ethanol fixation and staining, altering the accessibility of the DNA and reducing the resolution of the cell cycle profile.

C. Depletion of NASP dramatically aggravates the S phase defect resulting from Asf1 depletion. *Left*, Experimental scheme. U-2 OS cells were transfected first with Control or NASP siRNAs, then with Control or Asf1a+b siRNAs, and then either subjected to a double thymidine block or not and harvested for flow cytometry or preparation of soluble extracts. *Right*, Cell cycle profiles (as in B) of asynchronous cells or cells subjected to a double thymidine block and harvested while still in the second block, 48 h after transfection with Asf1 siRNA.

Figure 5. NASP depletion impairs buffering of soluble H3 upon acute replication stress.

Top, Experimental scheme. Cells transfected with Control or NASP siRNAs were subjected to a single thymidine block and then released into S phase for 4 h to obtain a synchronous mid S phase population. Cells were then optionally treated for 1 hour with HU to block replication. The cells (with or without HU treatment, “HU” and “S” respectively) were collected for flow cytometry (Figure S5A) or the preparation of soluble extracts and, *Left*, analyzed for NASP and H3 levels by Western blotting. Representative Westerns of four independent experiments where a 2x dilution series of each extract was loaded is shown. *Right*, Quantification of the ratio of soluble H3 in HU-treated versus normal S phase extracts for siControl or siNASP-transfected cells from three independent experiments. Quantification performed as in Figure S1E and Experimental Procedures.

Figure 6. NASP protects soluble H3-H4 from degradation by chaperone-mediated autophagy.

A. Scheme depicting proteasome and chaperone-mediated autophagy pathways for targeted protein degradation in mammalian cells and experimental methods for their blockade.

B. Proteasome inhibition cannot rescue the loss of soluble H3 in NASP-depleted cells. Western blot analysis of soluble extracts from HeLa S3 cells transfected with Control or NASP siRNAs and treated for 6 h with the proteasome inhibitor MG-132 (20 μ M) or DMSO prior to protein extraction. Increased β -catenin levels are shown as a positive control for proteasome inhibition by MG-132. Two different dilutions (1x, 3x) of each soluble extract were loaded.

C. Depletion of Hsp90 β or Hsc70 can completely rescue the loss of soluble H3-H4 in NASP-depleted cells. HeLa S3 cells were transfected first with Control or NASP siRNAs and then with siRNAs targeting either Luciferase (as non-targeting control), Hsp90 β or Hsc70. 72 h later, soluble extracts were prepared and analyzed as in B. Sample dilutions: 1x, 3x, 9x.

D. Depletion of Lamp-2a can completely rescue the loss of soluble H3-H4 in NASP-depleted cells. HeLa S3 cells were transfected with siRNAs in two rounds as described in A and extracts prepared 48 h later for analysis. Sample loading: 1x, 3x.

Figure 7. Working model: NASP maintains a soluble H3-H4 pool by protecting them from degradation by CMA.

NASP stabilizes a reservoir of soluble histones H3-H4 by protecting them from degradation *via* chaperone-mediated autophagy involving Hsc70 and Hsp90. Disposable for normal proliferation, NASP becomes important for S phase progression when limited Asf1 impacts upon proper H3-H4 provision to deal with an over-load of new H3-H4 and sudden H3-H4 accumulation during replication stress. Thus, fine-tuning of a reservoir of soluble H3-H4 by NASP integrates contingency into the histone supply chain to cope with unanticipated changes in demand or provision.

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NASP maintains a soluble pool of histones H3.1/H3.3-H4

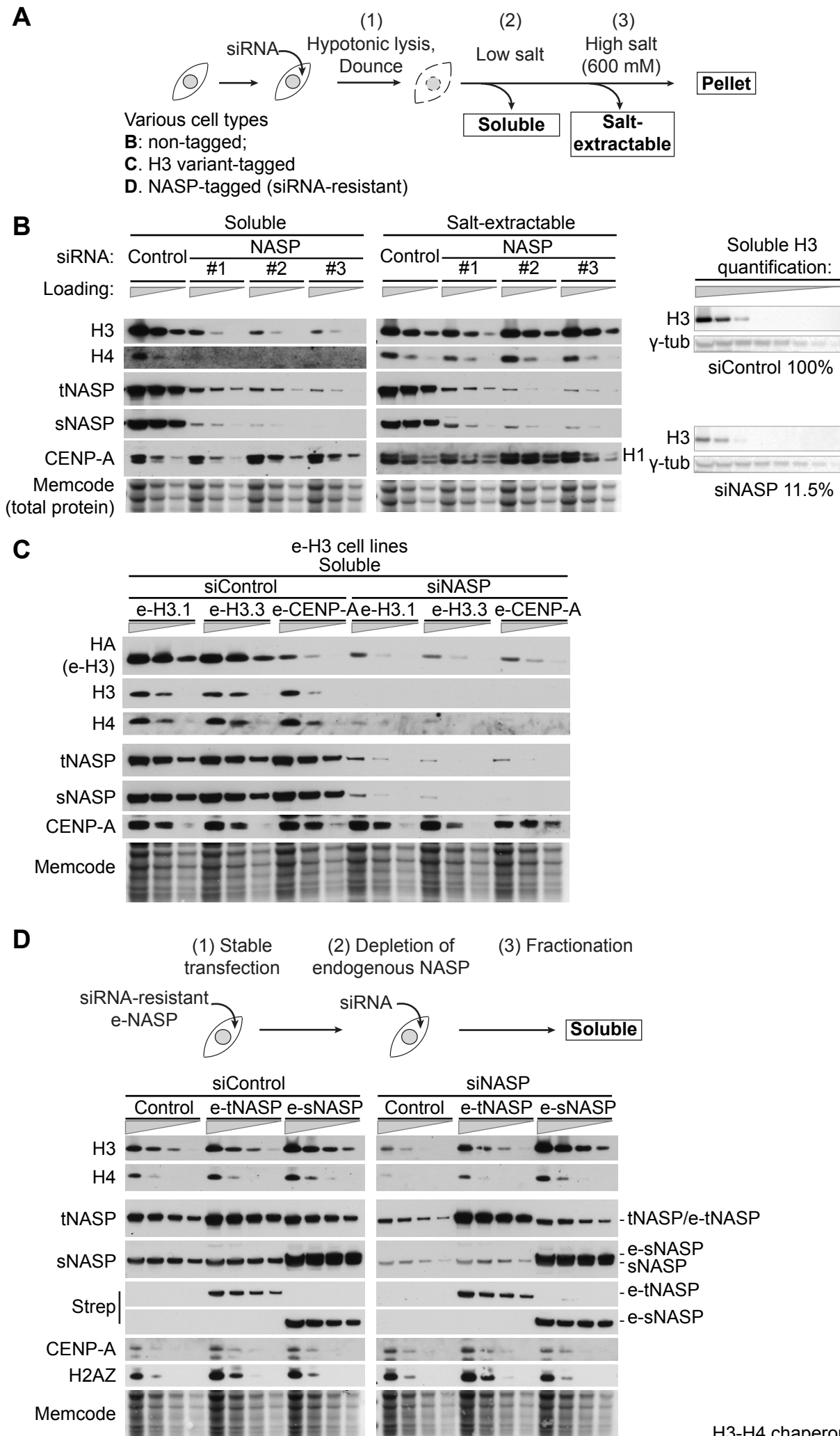
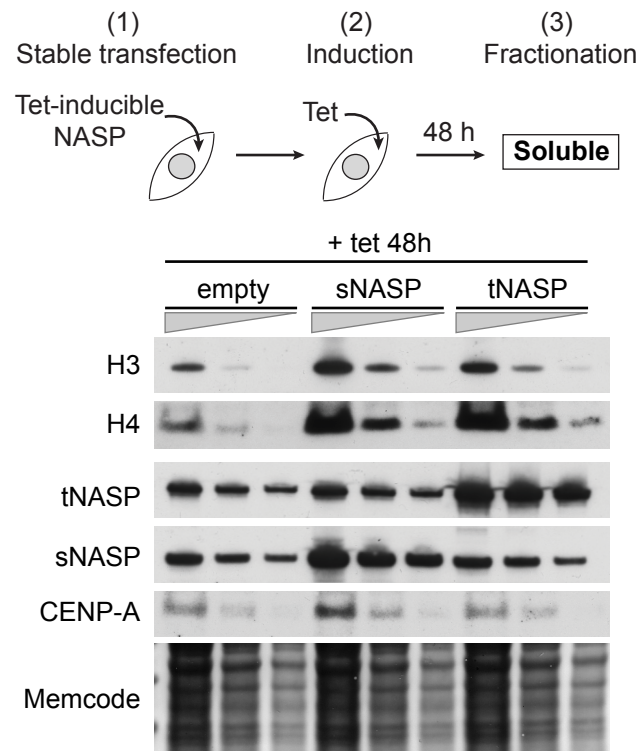


Figure 2
NASP over-expression increases the soluble H3-H4 pool



NASP interacts with the N-terminus of H3 and is unique to maintain a soluble pool of H3-H4

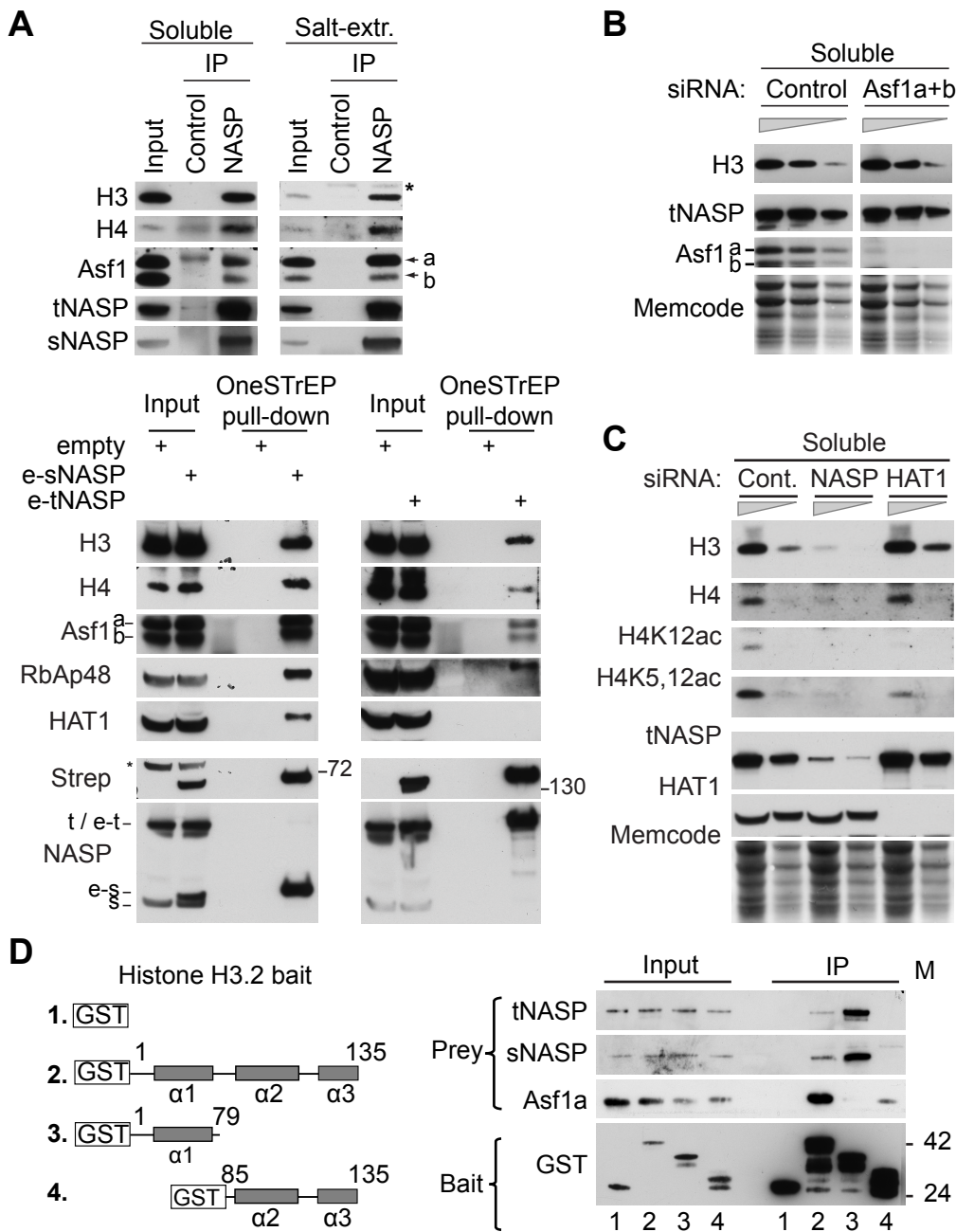


Figure 4
NASP affects the cellular response to perturbed histone supply

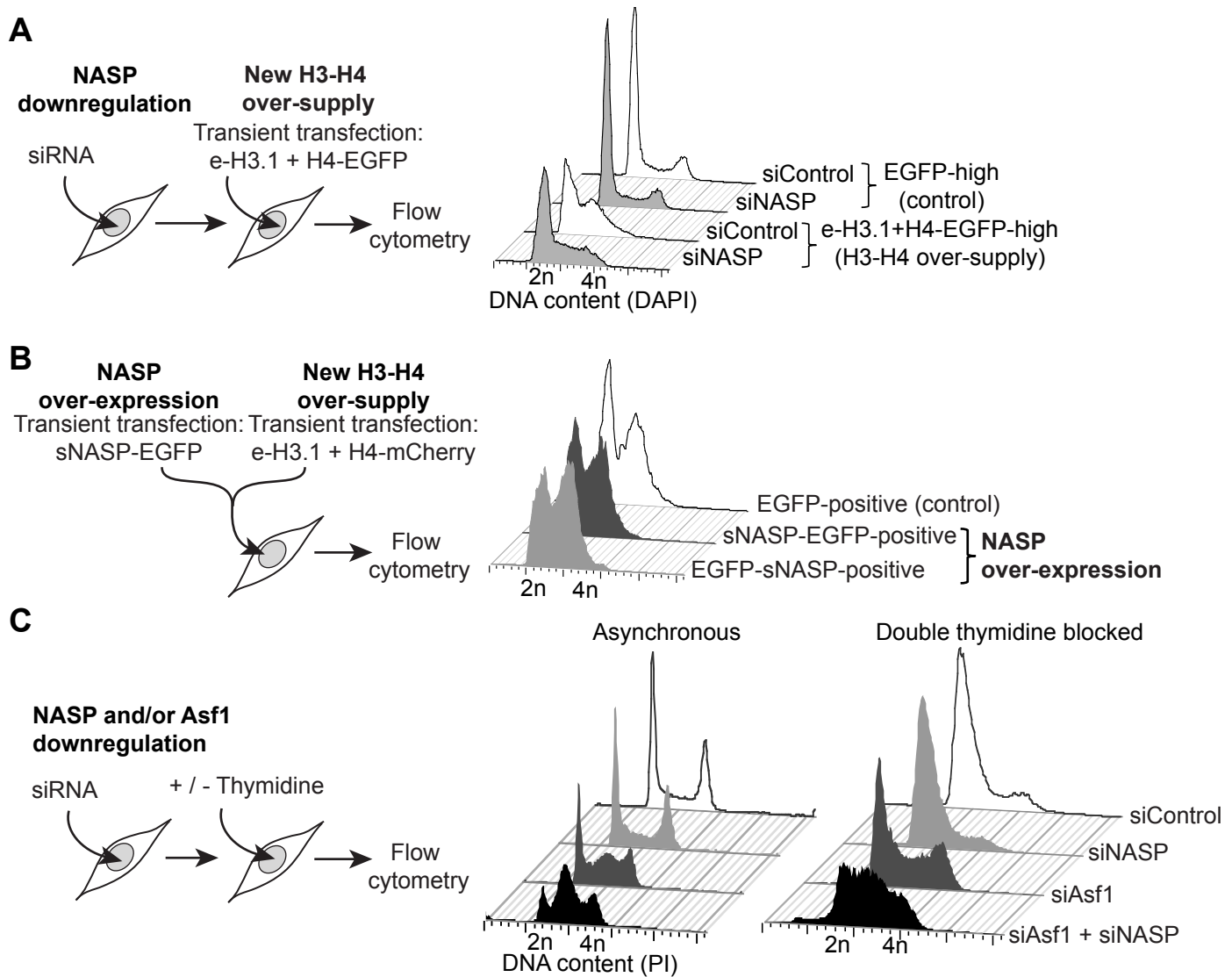
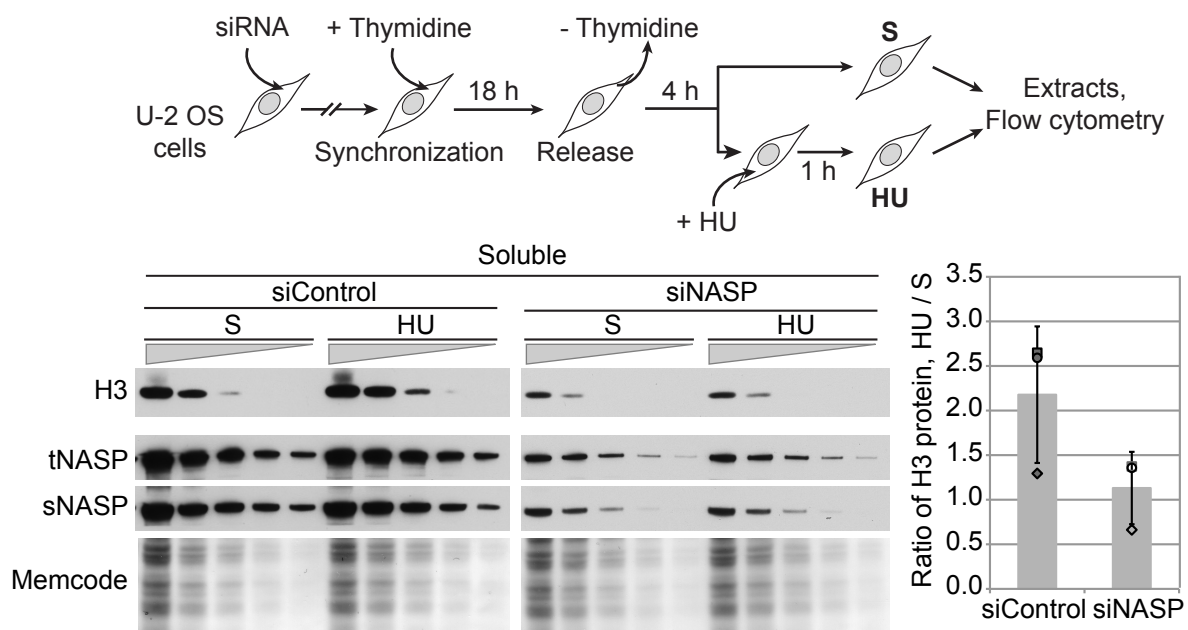


Figure 5
NASP depletion impairs buffering of soluble H3 upon acute replication stress



NASP protects soluble H3-H4 from degradation by chaperone-mediated autophagy

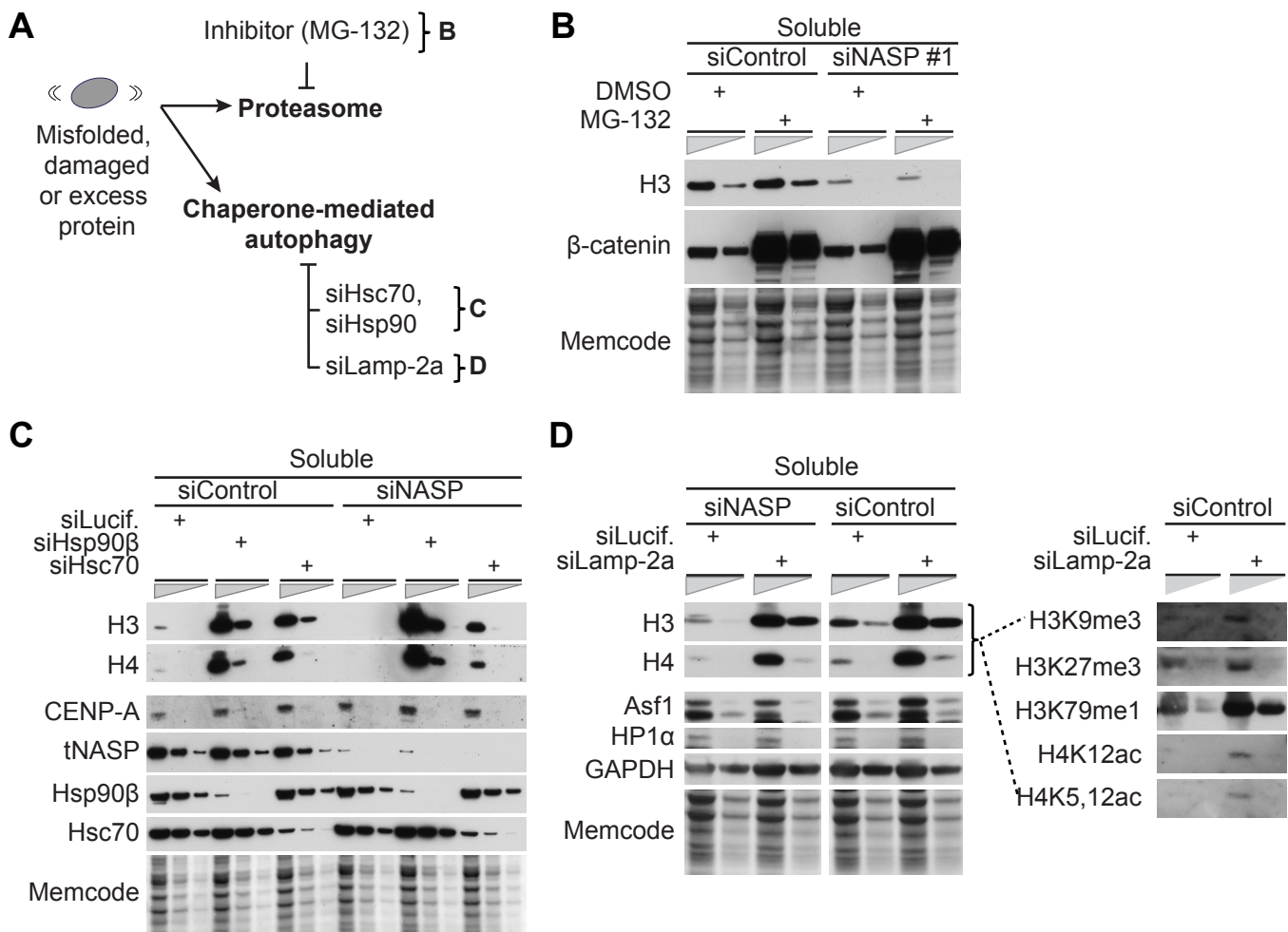
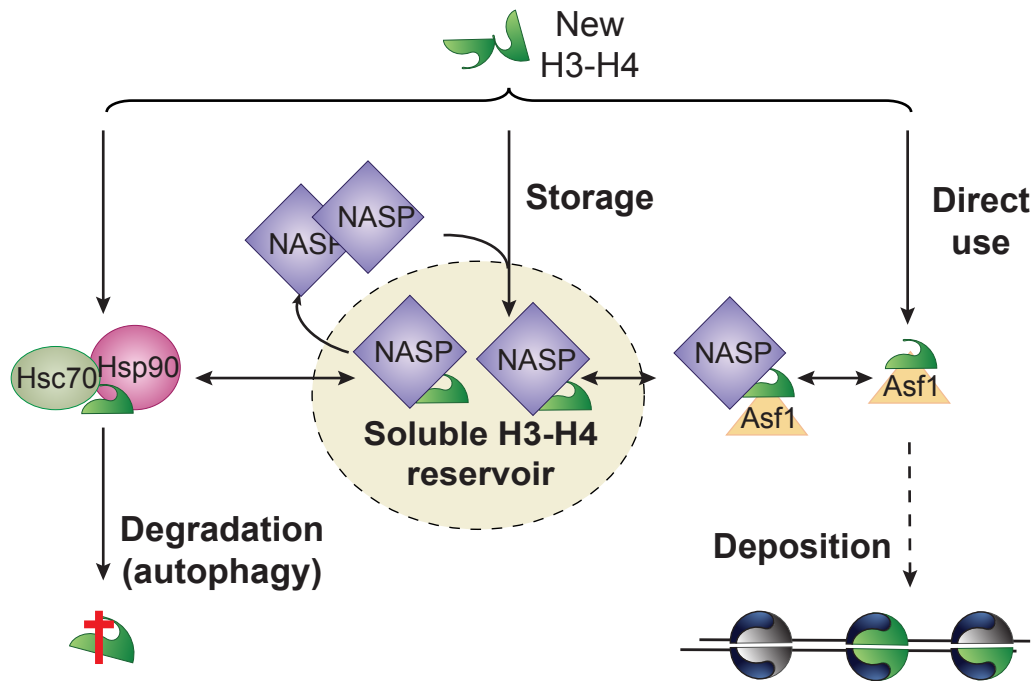


Figure 7

Working model: NASP maintains a soluble H3-H4 pool by protecting them from degradation by CMA



A specific function for the histone chaperone NASP to fine-tune a reservoir of soluble H3-H4 in the histone supply chain

Adam JL Cook^{1,2}, Zachary A Gurard-Levin^{1,2}, Isabelle Vassias^{1,2} and Geneviève Almouzni^{1,2,†}

¹Institut Curie, Centre de Recherche, Paris, F-75248 France;

²CNRS, UMR218, Paris, F-75248 France;

[†]to whom correspondance should be addressed:

genevieve.almouzni@curie.fr, +33 (0)1 56 24 67 01

SUPPLEMENTARY DATA

Supplementary Experimental Procedures

Cell extract buffer additives.

Buffers for cellular fractionation were supplemented with: DTT (ICN), Glycerol 2-phosphate (Sigma), Sodium fluoride (Sigma), PMSF (Sigma #P7626), Leupeptin (Roche Diagnostics, #11034626001) and Pepstatin (Roche Diagnostics, #11524488001).

Supplementary antibodies.

Rabbit polyclonal anti-serum against CAF-1 p60 was previously described (*Groth et al., 2005; Green and Almouzni, 2003*). Histone H2A (Abcam #ab18255), α -tubulin (clone DM1A, Sigma #T9026), β -actin (clone AC-15, Sigma #A5441), H3K9me1 (Upstate #07-450), Hsp90 (Abcam #ab13494), RNA Pol II phospho Ser5 (clone CTD4H8, Millipore #05-623), Lamin A (Cell Signalling #2032), Clathrin (Affinity Bio Reagents #MA1-065), SLBP (Abnova #7884).

NASP IP and pull-down of OneSTrEP-tagged NASP complexes.

For immunoprecipitation of NASP complexes, soluble or salt-extractable extracts (250 μ g) were first incubated with unlabelled Protein A-Sepharose CL-4B (GE Healthcare), which had been blocked with BSA, and IP buffer (50 mM Tris.Cl pH7.5, 0.5% (v/v) NP-40, Complete protease inhibitor (no EDTA) (Roche), 5 mM sodium fluoride, 10 mM β -glycerophosphate) in the presence of 150 mM NaCl for 30 min at 4°C. Following centrifugation, the pre-cleared extract (the supernatant) was then incubated for 4 h at 4°C with Protein A-Sepharose pre-coupled to NASP anti-serum (or pre-immune serum for control IP) to bind NASP complexes. Following washing with IP buffer containing 250 mM NaCl, the bound complexes were eluted by the addition of Laemmli sample buffer and analysed by SDS-PAGE.

For pull-down of OneSTrEP-tagged NASP complexes, soluble extracts prepared from 293 Flp-In cells stably expressing tagged sNASP or tNASP were first incubated with Superflow 6 resin (IBA) and IP buffer (50 mM Tris.Cl pH7.5, 0.1% (v/v) NP-40, 150 mM NaCl, Complete protease inhibitor (no EDTA) (Roche), 5 mM sodium fluoride, 10 mM β -glycerophosphate) for

30 min at 4°C. Following centrifugation, the pre-cleared extract (the supernatant) was then incubated for 3 h at 4°C with Strep-Tactin Superflow resin (IBA #2-1206-002) to bind NASP complexes. Following washing with IP buffer, the bound complexes were eluted in two steps (2 x 15 min) with IP buffer supplemented with 10 mM biotin (Sigma #B4501) and gentle shaking.

Recombinant proteins and *in vitro* interaction studies

NASP cDNAs were cloned into pET-30a(+) or pGEX-4T-1 plasmids for expression of His₆- and GST-NASP in *E. Coli*. GST-Asf1a and His-p60 were produced as previously described (Groth *et al.*, 2005). The expression, purification, and refolding of untagged histones (used in Fig S1D) were performed essentially as previously described (Clapier *et al.*, 2001), a slightly modified version from (Luger *et al.*, 1997; Luger *et al.*, 1999). For GST pull down, GST-tagged protein linked to glutathione Sepharose 4B (GE Healthcare) was incubated with purified proteins in binding buffer (20 mM Tris HCl pH 7.4, 600 mM NaCl, 1 mM MgCl₂, 1 % (v/v) NP-40, 0.5 mM DTT, 0.1% (v/v) Tween 20 and Complete protease inhibitor (no EDTA) (Roche)) for 2 hours at 4°C. After washing with binding buffer, bound proteins were eluted directly in SDS-PAGE loading buffer.

Proliferation assays and drug treatment

For the proliferation assay, cells were re-seeded 96 h after the first transfection, then analysed in triplicate at each timepoint with the ViCell (Beckman Coulter). Cell synchronisation experiments were performed either by: blocking once with 2 mM hydroxyurea for 18 h followed by release in fresh normal medium; a double thymidine block consisting of 2 mM thymidine (16 h), release in fresh medium supplemented with 24 mM 2-deoxycytidine (8 h), thymidine (16 h) and final release with 2-deoxycytidine, or; a single thymidine block.

Quantitative RT-PCR analysis of histone mRNA levels

Total RNA was extracted using an RNeasy kit (Qiagen) according to the manufacturer's recommendations and was treated with TURBO DNA-free kit (Applied Biosystems) to remove contaminating DNA. First-strand cDNA synthesis was performed with 3 µg of RNA and

random primers using Superscript III (Invitrogen) according to the manufacturers' recommendations. Following the RT reactions, cDNA products were diluted ten times in distilled water, and 1 μ l of the diluted cDNAs was spiked into reactions containing Power SYBR Green master mix (Applied Biosystems) and the appropriate primers. Cycling was performed on a 7900HT real time PCR machine (Applied Biosystems). Differences between samples and controls were calculated based on the $2^{-\Delta\Delta CT}$ (Schmittgen and Livak, 2008) method normalized to GAPDH. RT-PCR reactions were performed in triplicate for each RNA from three independent siRNA transfection experiments.

Flow cytometry.

Cells were washed with cold PBA (PBS + 0.1% (w/v) bovine serum albumin (Sigma #A4503) and 0.02% (w/v) sodium azide), fixed in -20°C 70% ethanol and stored at 4°C overnight. Fixed cells were washed with cold PBA, incubated in PBA + 100 μ g/mL propidium iodide (Sigma) and 40 μ g/mL Rnase A (USB)) for 30 min at 37°C, then analysed on the FACSCalibur, LSRII (both BD) or Accuri C6 (Accuri) flow cytometers. Cells which were co-transfected with H3-H4 and NASP expression plasmids were subjected to an additional fixation step of 1% (w/v) paraformaldehyde for 20 min at room temperature prior to being fixed with ethanol and were stained as above with 1 μ g/mL DAPI in place of PI. In all cases, cells were gated as follows: FSC-SSC (live cells), exclusion of cell doublets (PI/DAPI-H vs PI/DAPI-A). Flow cytometry data was analysed in FlowJo software (Treestar Inc.).

RT-PCR oligonucleotide sequences

The following oligonucleotide primers for RT-PCR were designed using the Primer Express software (Applied Biosystems) and were purchased from Sigma Genosys.

Gene	mRNA sequence Accession #	Forward primer (5'→3')	Reverse primer (5'→3')
GAPDH	NM_002046	CAAGGCTGTGGGCAAGGT	GGAAGGCCATGCCAGTGA
HIST1H1C (H1.2)	NM_005325	GCCTCAAAGGTGGCTACAAAAAC	CGTGGCCTTTTTGAGCTTTTTA
H2AZ	NM_002106	GCTTCAGCGGAATTCGAAAT	GGCCTTTCCGGAGTCCTTT
HIST1H2AC (H2a/l)	NM_175065	AATGGTGACACCCCCGAGTA	TGCAACTAGCCGTGAGGAATG
HIST1H2BD (H2B/b)	NM_021063	ACGCTACGATGCCTGAACCT	CTTCTTGGAGCCCTTCTTTGG
H3F3A (H3.3a)	NM_002107	TAAAGCACCCAGGAAGCAAC	AGGGAAGTTTGCGAATCAGA
H3F3B (H3.3b)	NM_005324	AAGCTGCCCTTCCAGAGGTT	ACCTCAGGTCGGTTTTGAAATC
HIST1H3A (H3/a)	NM_003529	AGATCCGCCGTTATCAGAAGTC	CAGGCGCTGGAAAGGTAGTT
HIST1H3D (H3/b)	NM_003530	ATCCGCCGCTACCAGAAGT	ACGCTGGAATGGCAGTTTG
HIST1H3E (H3/d)	NM_003532	GCGTGACCATCATGCCTAAAG	CCCTCTCCCCACGAATGC
HIST2H3C (H3/m)	NM_021059	GGGCTGTTCGAAGACACGAA	GGATGTCCTTGGGCATAATGG
HIST1H3B (H3/l)	NM_003537	GCTACCAAAAGTCGACCGAGTT	GCACCAGGCGCTGGAA
HIST1H3H (H3/k)	NM_003536	ATTCGCCGCTACCAGAAGTC	ACGCTGAAAAGGCAGCTTTC
HIST1H4E (H4/j)	NM_003545	GCGGAAAGGGACTGGGTAAA	TGTTATCTCGCAGGACCTTACG
HIST1H4A (H4/n)	NM_003548	GGATCTCTGGCCTCATTTACGA	CGAATCACATTCTCCAGGAACA
HIST4H4 (H4/p)	NM_175054	TGGGCAGCCCGATTTTC	CCTCGCCCAGACATTCTGA
CENP-A	NM_001809	GCCCTATTGGCCCTACAAGAG	GGCGTCCTCAAAGAGATGAACT
NASP	NM_152298.2	GCTGCCCAGGCACATCTTA	CCACAGCTTGACATAGTTTTCA

Supplementary Tables

Supplementary Table 1. Quantification of the level of histone H3 in cytosolic extracts from NASP-depleted compared to control cells. See Supplementary Figure 1E and Materials & Methods for details.

Experiment number	H3_{siNASP}% of control
AC1169	5.7
AC1174	10.0
AC1188	21.5
AC1192	11.5
Mean	12.2

Supplementary Figure Legends

Figure S1. Reduction of the soluble—but not the salt-extractable—pool of H3-H4 by siRNA-mediated depletion of NASP.

A. NASP is principally present in the soluble and salt-extractable extracts which contain non-nucleosomal histones. Cellular proteins were fractionated as previously described (Martini *et al.*, 1998) into “soluble” (containing cytoplasmic and some nuclear proteins), “salt-extractable” (principally nuclear proteins) and insoluble pellet fractions (see scheme in Figure 1A). The protocol is based on one which has been used extensively for the utility of the soluble extract in driving DNA replication *in vitro* with the SV40 large T antigen (Li and Kelly, 1984). It was exploited by Stillman and colleagues, who coined the terms cytosolic and nuclear to describe the respective fractions (Stillman and Gluzman, 1985), showing that the cytosolic extract can drive DNA synthesis-coupled nucleosome assembly when completed with CAF-1 p150 (Smith and Stillman, 1989). Fractions obtained from U-2 OS cells were analyzed by SDS-PAGE and Western blotting to assess the distribution of each protein between the cellular fractions. Aliquots of each extract corresponding to an equal number of cells (1x) and a dilution series thereof (2x for soluble and salt-extracted, 10x, 100x and 200x for the pellet) were loaded on the gel. The positions of the protein marker (Mk) bands of different molecular weights (kDa) are indicated on the right.

B. NASP siRNAs efficiently deplete total cellular NASP. *Top*, U-2 OS cells were transfected twice with NASP or Control siRNAs and total cellular protein solubilized directly in loading buffer to generate a whole-cell extract. SDS-PAGE and Western blotting was performed with NASP anti-serum. Equal loading is confirmed by α -tubulin staining. *Bottom*, U-2 OS cells transfected with siRNAs as above, fixed and labeled with NASP anti-serum and analyzed by fluorescence microscopy. Scale bar, 10 μ m.

C. NASP depletion does not affect the level of H3.1 or H3.3 in HeLa salt-extractable extracts. Extracts were prepared from an siRNA-transfected HeLa cell line stably expressing epitope-tagged H3.1, H3.3 or CENP-A and analyzed as for soluble extracts in Figure 1D.

D. siRNA-mediated depletion of NASP does not affect the level of soluble H2A. Soluble extracts from U-2 OS and HeLa S3 cells transfected with Control and NASP siRNAs were analyzed by SDS-PAGE and Western blotting. Sample dilutions: 1x, 3x.

E. Method for calculating the difference in soluble H3 between NASP-depleted and control cells. A 2x dilution series of soluble extracts from siControl and siNASP-transfected U-2 OS cells were separated by SDS-PAGE, Western blotted with an H3 specific antibody and revealed with a chemiluminescent substrate. Chemiluminescent signals were acquired on the Chemi-doc system and analyzed using the QuantityOne software (both from Bio-Rad). H3 signal intensities for each dilution series were graphed in Microsoft Excel and lines of best linear fit drawn. If the lines-of-best fit yielded an R-squared value greater than 0.99, the ratio of the gradients was calculated to determine relative H3 levels in NASP-depleted extracts with respect to Control extracts.

F. Quantitative RT-PCR analysis of histone mRNA levels. Total RNA was isolated from U-2 OS cells transfected twice with NASP or Control siRNAs and analyzed by quantitative reverse-transcription (RT)-PCR. Individual data points represent the fold-change in transcript levels in siNASP-transfected cells compared to siControl-transfected cells from each of three independent biological replicate experiments. Also shown are the Mean fold-changes (columns) +/- SEM for the three experiments. Individual histone genes are, in order: HIST1H1 (H1.2), HIST1H3D (H3/b), HIST1H3A (H3/a), HIST1H3B (H3/l), HIST1H3E (H3/d), HIST1H3H (H3/k), HIST2H3C (H3/m), H3.3(A) (H3.3a), H3.3(B) (H3.3b), CENP-A, HIST1H4E (H4/j), HIST1H4A (H4/n), HIST4H4 (H4/p), HIST1H2AC (H2A/l), H2AZ, HIST1H2BD (H2B/b).

G. Viable NASP-depleted U-2 OS cells accumulate normally in culture. Cells were transfected twice with Control or NASP siRNA and at 96 h following the 1st transfection, equal numbers were re-plated. At 24 h intervals following re-plating. Triplicate samples of the cells transfected with each siRNA were collected for subsequent analysis. *Top*, The number of live cells at each time-point were counted using the ViCell robot and graphed as the log of the number of cells re-plated at the start of the assay. *Bottom*, Western blotting confirming sustained knock-down of NASP over the course of the experiment.

H. Depletion of NASP does not alter the cell cycle profile. U-2 OS cells were transfected with Control or NASP siRNAs for 120 h and were collected, fixed, stained with propidium iodide (PI) for analysis of cell cycle distribution by flow cytometry.

I. NASP-depleted and control cells progress similarly through S phase. *Top*, Experimental scheme. U-2 OS cells were transfected with siRNA (as before) and then 120 h after the 1st siRNA transfection were synchronized at the G1/S border of the cell cycle by either hydroxyurea (HU) or a double thymidine block. Following release from the block (120 h after 1st siRNA transfection), cells were collected at various times (as indicated) for fixation and staining with PI. *Bottom*, The distribution of the cells through the cell cycle was revealed by flow cytometric analysis of PI staining.

Figure S2. Controls for the effect of NASP over-expression on the soluble H3-H4 pool.

A. NASP over-expression does not affect the cell cycle. Flp-In cell lines stably carrying tNASP or sNASP transgenes whose expression can be induced by treatment with tetracycline (tet) were treated for 48 h or not with tetracycline and harvested, fixed, stained with PI and analyzed by flow cytometry.

B. Soluble and salt-extractable extracts were prepared from cells treated as in A. and a 2x dilution series of each extract was analyzed by SDS-PAGE and Western blotting. Note that H3-H4 levels are not altered in soluble or salt-extractable fractions from non-induced NASP cell lines with respect to the control cell line. See also Figure 2.

Figure S3. NASP interacts with H3 independently of Asf1.

NASP preferentially interacts with histone H3 *in vitro* and this can promote its association with Asf1. *Top*, GST-human NASP fusion proteins or unfused GST were expressed in *E. coli* and bound to Glutathione sepharose resin as bait for subsequent incubation with core recombinant histones H3.1, H4, H2A or H2B and pull-down. Binding of each histone was assessed by SDS-PAGE and Western blotting with specific antibodies. *Bottom*, Recombinant GST-human Asf1a fusion proteins or unfused GST were expressed in *E. coli* and pre-bound to Glutathione sepharose resin and then incubated with recombinant His₆-tagged sNASP or tNASP, in the

presence or absence of untagged H3.1, or His₆-tagged human p60. Bound proteins were analyzed by SDS-PAGE and Western blotting with NASP anti-serum or antibodies against H3 or p60. Input, 10%.

Figure S4. Controls for the effect of NASP when histone supply is perturbed.

A. NASP depletion reduces the level of soluble e-H3.1 expressed from a transiently transfected plasmid. NASP or Control siRNA-transfected U-2 OS cells were transfected with plasmids from which either EGFP or e-H3.1+H4-EGFP is expressed. Soluble extracts prepared from the cells were diluted in a 2x series and analyzed by Western blotting to determine the effect of NASP depletion on the level of e-H3.1. See also Figure 4A.

B. NASP over-expression does not alter the cell cycle in cells with a low over-supply of new H3-H4. U-2 OS cells were co-transfected with a plasmid to co-express e-H3.1 and H4-mCherry or EGFP alone (control) and a plasmid to express sNASP fused to EGFP (at the C or N terminal, sNASP-EGFP or EGFP-sNASP, respectively). Thirty hours following transfection the cells were harvested for flow cytometry. Cells were fixed with paraformaldehyde (to retain the GFP/mCherry fusion proteins), prior to ethanol fixation and staining with DAPI. Flow cytometry analysis of the cell cycle profiles of cells gated for moderate EGFP expression (moderate expression of sNASP-EGFP fusion protein or EGFP control) and low mCherry intensity (low over-supply of e-H3.1 and H4-mCherry). See also Figure 4B.

C. Confirmation of siRNA-mediated depletion of NASP and/or Asf1. Representative Western blot analysis of a 2x dilution series of soluble extracts from siRNA-transfected U-2 OS cells analyzed by flow cytometry in Figure 4C.

Figure S5. Buffering of soluble H3-H4 is impaired by NASP depletion.

A. U-2 OS cells released from the thymidine block in Figure 5 are mostly in S phase. For the study of HU-induced buffering of soluble H3-H4, cells were transfected with siRNAs, synchronized with thymidine and released into S phase for 4.5 hours as described in Figure 5. Cells were harvested, fixed and stained with PI prior to analysis of their DNA content by flow cytometry.

B. HU-induced accumulation of H3 in the soluble pool in S phase cells is impaired by the depletion of NASP. As in Figure 5, soluble and salt-extractable extracts were prepared from control (S) or stressed (HU) S phase cells transfected with Control siRNA or siRNA targeting NASP. To determine the relative levels of histone H3 (according to method described in Figure S1E) and additionally to correct for any differences in sample loading using γ -tubulin staining, an extended dilution series of each extract was analyzed by SDS-PAGE and Western blotting so that lines-of-best fit with R-squared values > 0.99 could be fit for both H3 and γ -tubulin.

C. Control Western blots showing histone levels in chromatin fractions obtained as in B.

Figure S6. NASP protects soluble H3-H4 from degradation by chaperone-mediated autophagy.

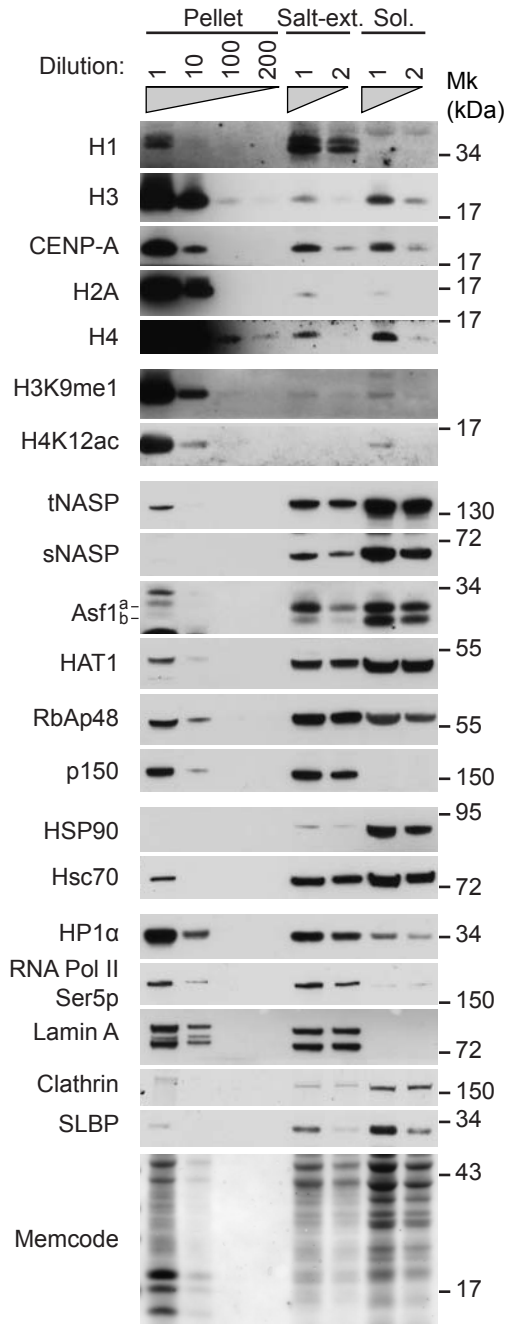
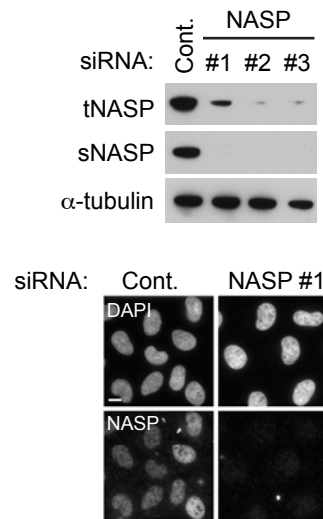
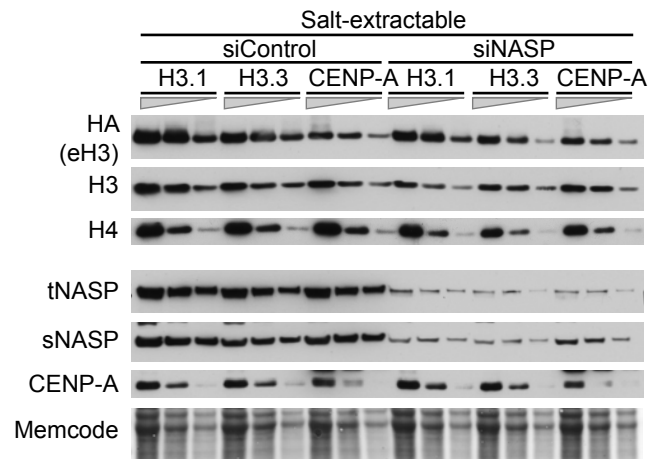
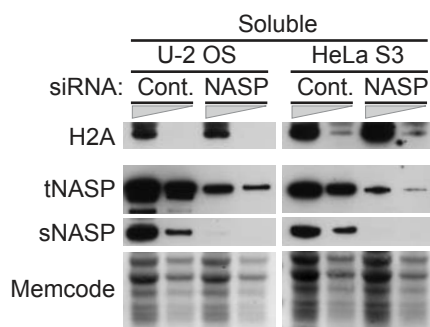
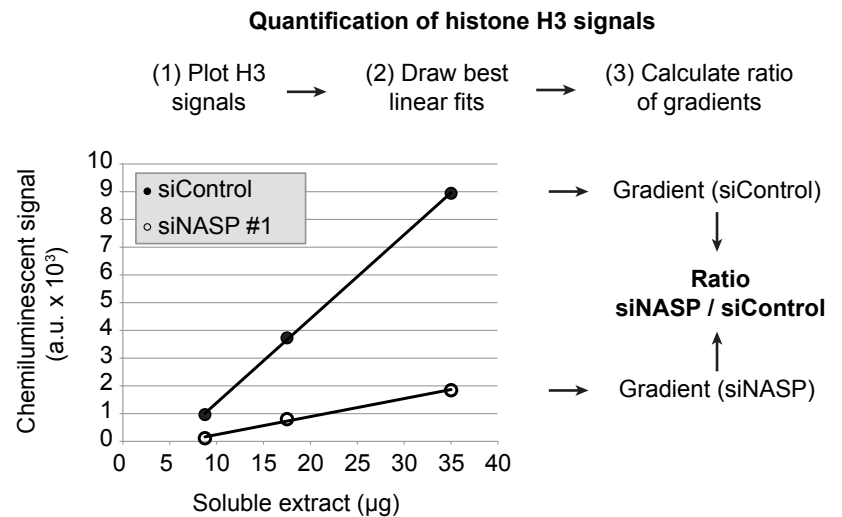
A. Hsp90 β depletion does not non-specifically increase the level of soluble proteins. Soluble extracts prepared from HeLa S3 cells transfected with siLuciferase (as control) or siHsp90 β were analyzed by SDS-PAGE and Western blotting with specific antibodies. Two sample dilutions (1x, 3x) were loaded on the gel.

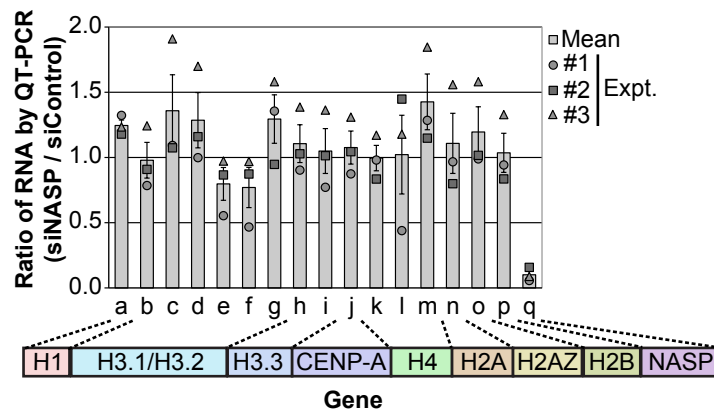
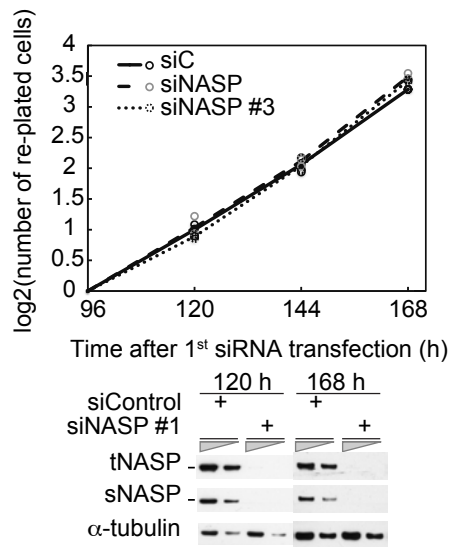
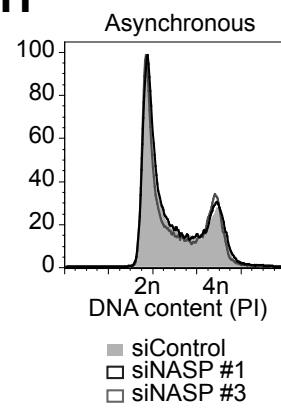
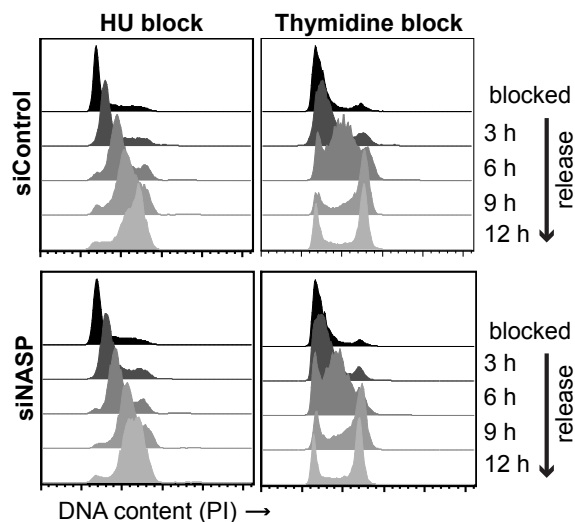
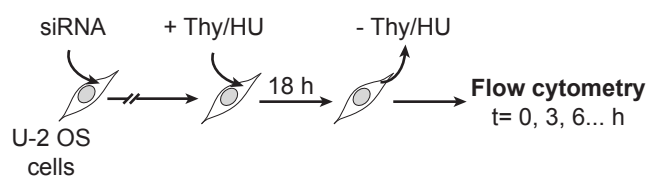
B. Control Western blots of whole-cell extracts transfected with siLamp-2a showing that total histone levels are not affected. HeLa S3 cells transfected with siControl or siNASP and subsequently siLuciferase (as control) or siLamp-2a were solubilized directly in Laemmli sample buffer and analyzed by SDS-PAGE and Western blotting with specific antibodies. Two dilutions (1x, 3x) of each sample were loaded on the gel.

C. Cell cycle analysis of cells transfected with Hsp90 β , Hsc70 or Lamp-2a siRNAs.

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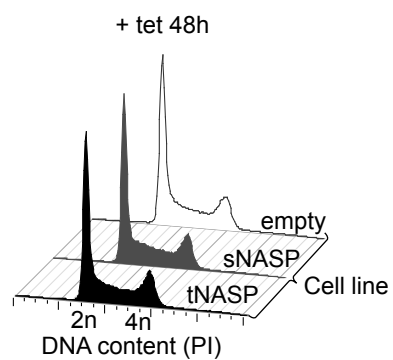
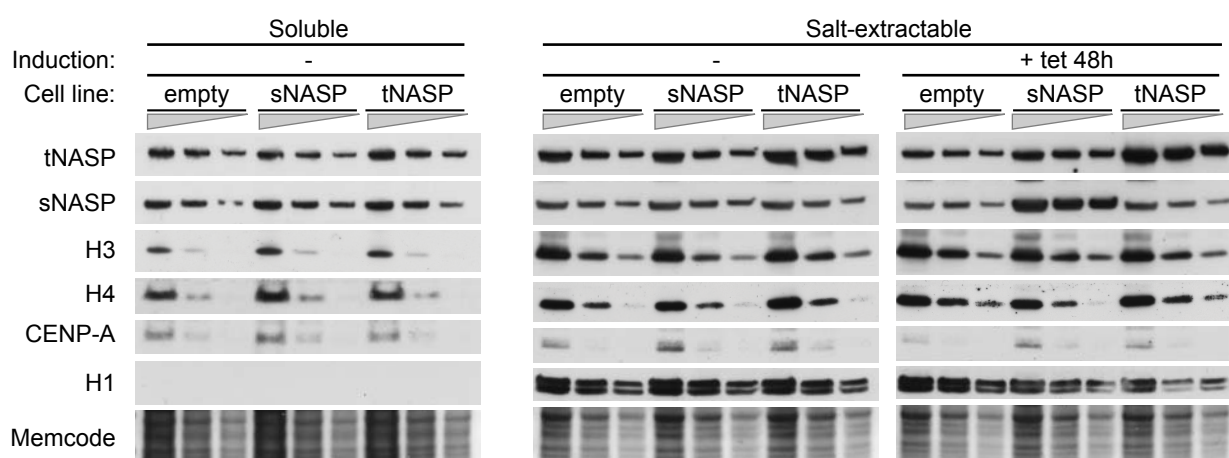
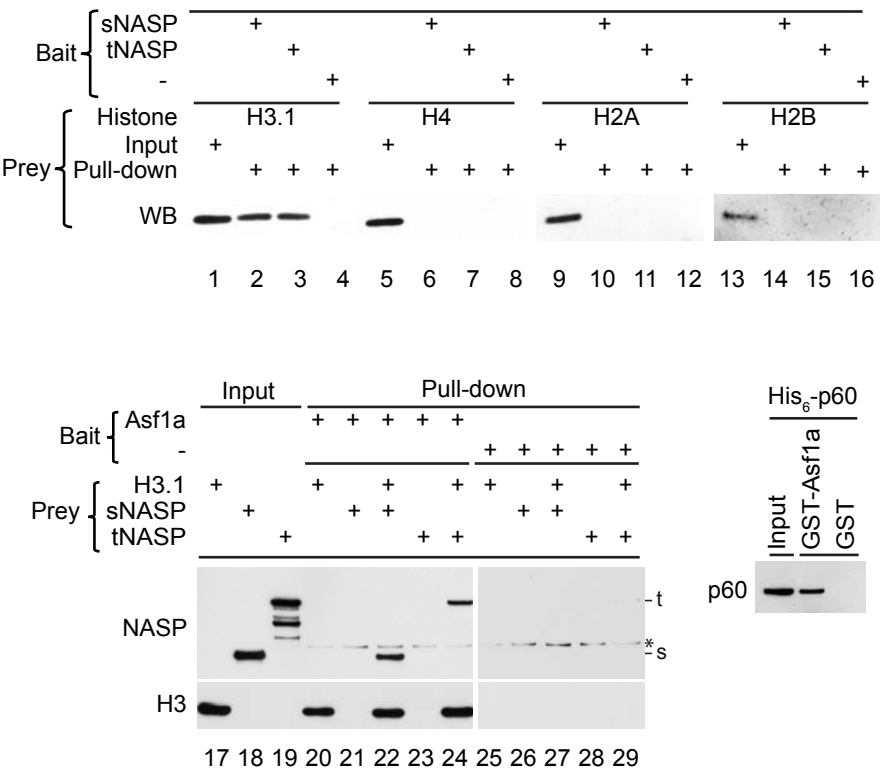
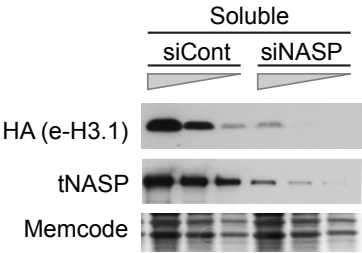
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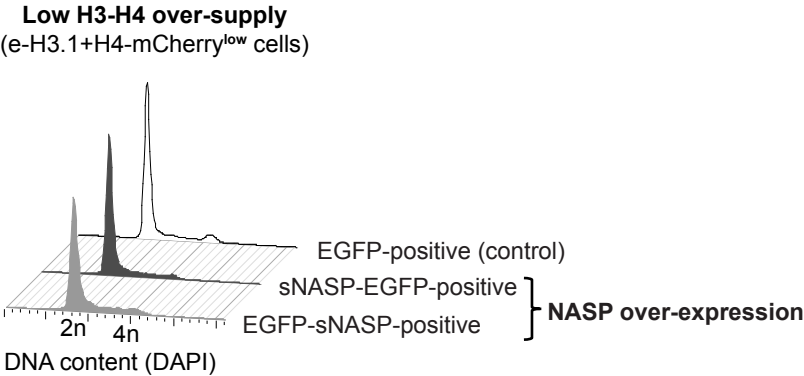
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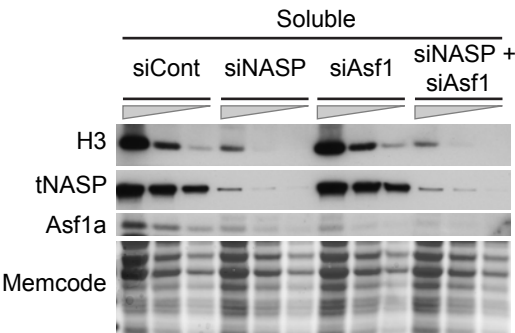
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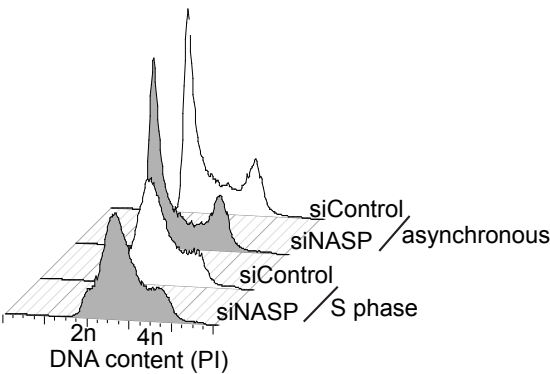
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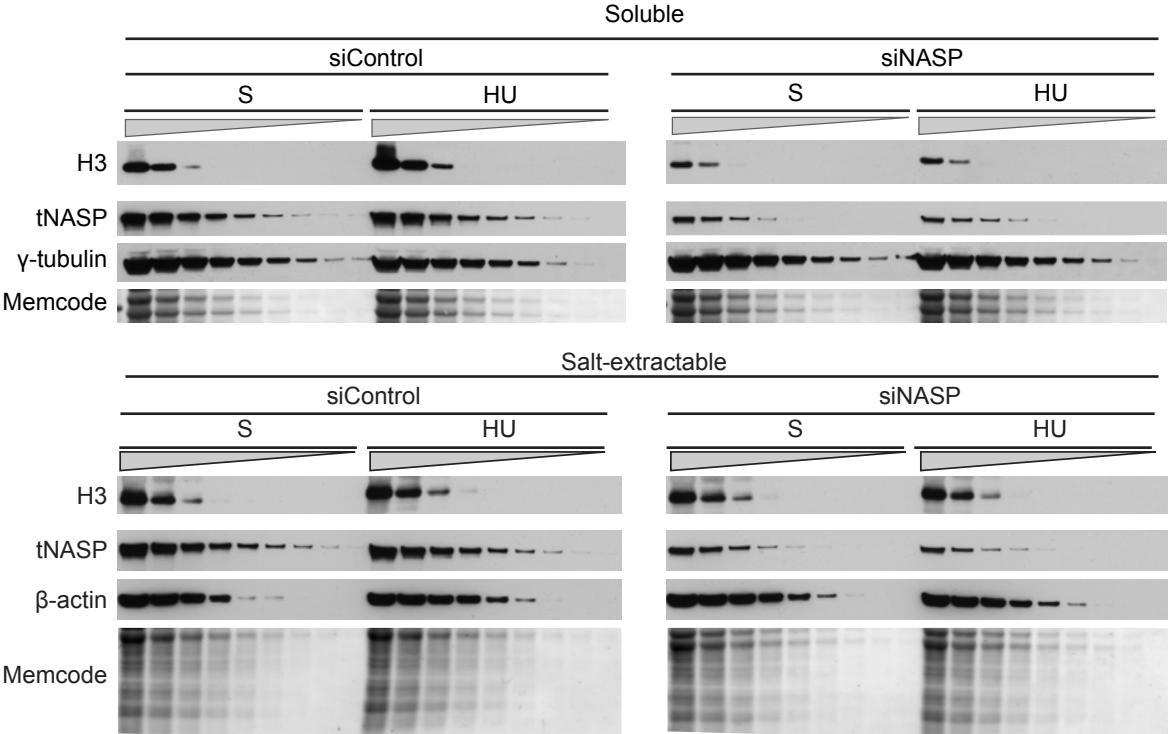
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