Title

Ataxin-7 and Non-stop coordinate SCAR protein levels, subcellular localization, and actin cytoskeleton organization

Running Title

SCAR is regulated by Ataxin-7 and Non-stop

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Summary

SAGA subunits Ataxin-7 and Non-stop regulate stability and subcellular localization of WRC subunit SCAR. Loss of Ataxin-7 increases, while loss of Non-stop decreases, SCAR protein levels and F-actin network assembly.

Abstract

Ataxin-7 (Atxn7), a subunit of the SAGA transcriptional coactivator complex, is subject to polyglutamine expansion at the amino terminus, causing spinocerebellar ataxia type 7 (SCA7), a progressive retinal and neurodegenerative disease. Within SAGA, the Atxn7 amino terminus anchors the Non-stop deubiquitinase to the complex. To understand the consequences of Atxn7-dependent regulation of Non-stop, we sought substrates for Non-stop and discovered the deubiquitinase, dissociated from SAGA, interacts with Arp2/3 and WAVE regulatory complexes (WRC). Protein levels of WRC subunit SCAR are regulated by a constant ubiquitination/proteasomal degradation mechanism. Loss of Atxn7 frees Non-stop from SAGA, and increased protein levels of WRC subunit SCAR, while loss of Non-stop resulted in reduced protein levels of SCAR. Proteasome inhibition fully rescued SCAR protein levels upon loss of Non-stop. Dependent on conserved WRC interacting receptor sequences (WIRS), Non-stop

overexpression increased SCAR protein levels and directed subcellular localization of SCAR, leading to decreased cell area and decreased number of protrusions.

Keywords: Ataxin-7, Non-stop, USP22, Arp2/3, spinocerebellar ataxia type 7, SCA7, actin, WAVE regulatory complex, WRC, SAGA complex, SCAR, neural.

Introduction

The Spt Ada Gcn5 Acetyltransferase (SAGA) transcriptional coactivator complex is an approximately 1.8 MDa, modular, multi-protein complex, bearing two enzymatic subunits (Gcn5 acetyltransferase and Non-stop deubiquitinase) housed in separate modules of the complex (Lee et al., 2011a). Within SAGA, the Ataxin-7 (Atxn7) amino-terminus anchors the deubiquitinase module (DUBm) to the larger complex (Lee et al., 2009). Mutation of *Atxn7* and *non-stop* leads to aberrant gene expression, defective non-homologous end joining, decreased immune response, reduced replicative lifespan, neurodegeneration, blindness, tumorigenesis, and cancer (Bonnet et al., 2014; Furrer et al., 2011; Glinsky et al., 2005; Lang et al., 2011; Li et al., 2018; McCormick et al., 2014; Weake et al., 2008).

The progressive retinal and neurodegenerative disease Spinocerebellar Ataxia type 7 (SCA7) is caused by CAG trinucleotide repeat expansion of the *ATXN7* gene, resulting in polyglutamine (polyQ) expansion at the amino terminus of the Atxn7 protein (David et al., 1997; Giunti et al., 1999). SCA7 disease is characterized by progressive cone-rod dystrophy leading to blindness, and progressive degeneration of the spine and cerebellum (Garden, 1993; Martin, 2012).

In *Drosophila*, loss of *Atxn7* leads to a phenotype similar to overexpression of the polyQ-expanded amino terminal truncation of human Atxn7 (Latouche et al., 2007) – reduced life span, reduced mobility, and retinal degeneration (Mohan et al., 2014b). Without Atxn7, the Drosophila DUBm is released, enzymatically active, from SAGA. Once released, the module acts with a gain-of-function, leading to reduced ubiquitination of H2B. Similarly, the mammalian DUBm binds and deubiquitinates substrates without Atxn7, albeit at a lower rate *in vitro* (Lan et al., 2015; Yang et al., 2015). Consistent with Non-stop release and over activity in mediating the Drosophila Atxn7 loss-of-function phenotype, reducing *non-stop* copy number alleviates lethality associated with loss of Atxn7 (Mohan et al., 2014b).

Non-stop is a critical mediator of retinal axon guidance and important for glial cell survival (Martin et al., 1995; Weake et al., 2008). Interestingly, abnormal ubiquitin signaling in

the nervous system contributes to a number of genetic and spontaneous neurological and retinal diseases, including SCA3, SCAR16, Alzheimer's, Parkinson's, ALS, and Huntington's disease (Campello et al., 2013a; Campello et al., 2013b; Mohan et al., 2014a; Petrucelli and Dawson, 2004). Few functions are known for the DUBm beyond deubiquitination of H2Bub, H2Aub, shelterin, and FBP1 (Atanassov and Dent, 2011; Atanassov et al., 2009). In polyQ-Atxn7 overexpression models, sequestration of the DUBm may result in reduced deubiquitinase activity on critical substrates. Interestingly, both increased and decreased H2Bub have been shown to hinder gene expression, and imbalances (whether up or down) in ubiquitination have been observed in numerous retinal and neurological diseases (Ristic et al., 2014). Together, these data suggest more needs to be understood about Atxn7-mediated regulation of DUBm function.

We set out to identify substrates for Non-stop and determine the consequence of Atxn7 loss on Non-stop/substrate interactions. To this end, we purified Non-stop-containing complexes, fractionated them by size, and tested enzymatic activity to identify novel complexes bearing enzymatically active Non-stop. This revealed a portion of functionally active DUBm associates with protein complexes distal from SAGA. Mass spectrometry revealed Arp2/3 complex and Wiskott-Aldrich syndrome protein (WASP)-family verprolin homologous protein (WAVE) Regulatory Complex (WRC) members SCAR, HEM protein (Hem), and specifically Rac1 associated protein 1 (Sra-1), as interaction partners of the independent DUBm. These complexes function together to initiate actin branching (Kurisu and Takenawa, 2009). Pull-down and immunofluorescence verified interaction and revealed extensive colocalization between Non-stop and WRC subunit SCAR.

Spatiotemporal regulation of SCAR is essential for maintaining cytoskeletal organization. A constant ubiquitination-proteasomal degradation mechanism contributes to establishing SCAR protein amount. WRC subunits HEM, SRA-1, and Abi mediate interaction with an unidentified deubiquitinase to counteract proteasomal degradation of SCAR (Kunda et al., 2003). WRC subunits Abi and SRA-1 mediate interaction with numerous proteins which bear a WIRS motif to regulate SCAR location. In cells and in flies, loss of Atxn7 resulted in a 2.5 fold increase in SCAR protein levels, suggesting released Non-stop counteracts proteasomal degradation of SCAR. Accordingly, loss of Non-stop led to a 70% loss of SCAR protein, which was completely rescued by proteasome inhibition. Overexpression of Non-stop increased total SCAR protein levels approximately 5-fold. Interestingly, SCAR protein levels increased in cellular compartments where Non-stop increased, including in the nucleus. The functional

output of SCAR is F-actin formation. When SCAR protein levels were increased and relocalized by augmenting Non-stop, local F-actin amounts increased too, leading to cell rounding and fewer cell protrusions. Analysis of Non-stop protein sequence revealed the presence of WIRS motifs. Mutation of these reduced Non-stop ability to increase SCAR protein, F-actin, and to reduce cellular protrusions.

Results

Non-stop interacts with multi-protein complexes distal from SAGA

Loss of Atxn7, releases Non-stop from SAGA resulting in a deubiquitinase gain-of-function, reducing ubiquitination of histone H2B (Mohan et al., 2014b). The resultant retinal and neurodegenerative phenotype is rescued by reducing *non-stop* yet it is not consistent with phenotypes arising from mutations in other regulators of H2B ubiquitination, such as Dhr6 (the E2 ubiquitin conjugating enzyme), Bre1 (E3 ubiquitin ligase), or USP7 (H2B deconjugating enzyme) (Haddad et al., 2013; Mohan et al., 2010; Tsou et al., 2012; van der Knaap et al., 2010; van der Knaap et al., 2005). Therefore, we sought novel interaction partners for Non-stop (Figure 1A). To this end, an unbiased biochemical screen was designed to specifically identify protein complexes stably interacting with the distal, enzymatically active, SAGA DUBm (Figure 1B). Drosophila S2 cells were stably-transfected with pRmHa3-based inducible expression vectors to produce epitope-tagged Atxn7-2xFLAG-2xHA (Atxn7-FH), or Non-stop-2xFLAG-2xHA (Non-stop-FH) (Bunch et al., 1988; Cherbas et al., 2011; Guelman et al., 2006; Kusch et al., 2004; Mohan et al., 2014b; Suganuma et al., 2008). Cells stably transfected with empty vector served as negative control and Atxn7-FH cells served as positive control since Atxn7 has been previously characterized by purification, gel filtration, and mass spectrometry (Mohan et al., 2014b).

Protein complexes containing either Non-stop or Atxn7 were purified from nuclear extracts via tandem purification of their epitope tags: FLAG, then HA (Suganuma et al., 2010). Isolated complexes were separated by size using Superose 6 gel filtration chromatography (Figure 1C) (Kusch et al., 2003). To identify fractions containing enzymatically active Non-stop, the ubiquitin-AMC deubiquitinase activity assay was performed, in which fluorescent probe AMC is initially quenched by conjugation to ubiquitin but fluoresces when released by deubiquitination, permitting an indirect measurement of the relative amount of deubiquitination occurring in each sample (Figure 1C) (Köhler et al., 2010; Samara et al., 2010a).

In complexes purified through Atxn7, peak deubiquitinase activity was detected around 1.8MDa, consistent with SAGA complex and previous purification, fractionation, and mass spectrometry of Atxn7 (Kusch et al., 2003; Lee et al., 2011b; Mohan et al., 2014b). In Non-stop purifications, however, deubiquitinase activity resolved into three major peaks. The major activity peak resolved about 1.8 MDa, along with SAGA, and was labeled “Group 1”. A secondary peak, “Group 2”, was resolved centering approximately 669 kDa, which was interpreted to be non-SAGA large multi-protein complexes. A final peak with the lowest enzymatic activity, centering about 75 kDa, was labeled “Group 3.” The DUBm, consisting of Non-stop, Sgf11, and E(y)2 are predicted to have a molecular mass of 88 kDa, meaning this last peak was the DUBm interacting with very small proteins, if any.

Three fractions comprising the center of each peak were combined, and MudPIT mass spectrometry performed to identify the protein constituents of each (Washburn et al., 2001). Consistent with previous characterizations of SAGA, Group 1 contained SAGA complex subunits from each of the major (HAT/TAF/SPT/DUB) modules (Kusch et al., 2003; Lee et al., 2011b; Mohan et al., 2014b; Setiaputra et al., 2015). Previous fractionation demonstrated that Atxn7 is present in group 1 alone (Mohan et al., 2014b). Groups 2 and 3 contained the DUBm: Non-stop, Sgf11, and E(y)2 – indicating the DUBm remained intact during purification and fractionation (Köhler et al., 2010; Lee et al., 2011b; Samara et al., 2010a). SAGA subunits characteristic of the larger complex, Nipped A (SPT module) and Ada2b (HAT module), were absent from groups 2 and 3 (Figure 1D). Histone H2A and H2B were detected in both groups 1 and 2, suggesting the DUBm was co-purifying with known substrates, and further confirming conditions were suitable for preservation and identification of endogenous interactions (Figure 2A) (Henry et al., 2003; Zhao et al., 2008). Furthermore, Non-stop distributed normalized spectral abundance (dNSAF) was not over-represented relative to Sgf11 and E(y)2, showing Non-stop bait protein was not expressed beyond physiological levels (Figure 2A) (Zhang et al., 2010).

Non-stop co-purifies with Arp2/3 and WRC complexes

MudPit proteomics provides a dNSAF factor which acts as a reporter for relative protein amount within a sample (Washburn et al., 2001). Notably, within Group 2, Arp2/3 complex (all subunits) and WRC subunits SCAR, Hem, and Sra-1 were represented in high ratio to DUBm members, as determined by dNSAF (Figure 2A) (Chen et al., 2010; Pollard and Beltzner, 2002).

WRC complex activates Arp2/3 to promote actin branching, which is important for a range of cellular activities including cell migration, cell adhesion, exocytosis, endocytosis, maintenance of nuclear shape, and phagocytosis (Alekhina et al., 2017; Navarro-Lerida et al., 2015; Takenawa and Miki, 2001; Verboon et al., 2015; Vishavkarma et al., 2014; Zallen et al., 2002). Non-stop and Atxn7-containing complexes were purified from nuclear extracts, where their function in gene regulation has been best characterized. WRC and Arp2/3 function in the nucleus is more enigmatic. Although Arp2/3 and WRC have demonstrated nuclear functions, their roles in actin branching in the cytoplasm are better understood (Miyamoto et al., 2013; Navarro-Lerida et al., 2015; Rawe et al., 2004; Verboon et al., 2015; Yoo et al., 2007). SAGA, WRC, and Arp2/3 complexes are all critical for proper central nervous system function, making the regulatory intersection for these complexes intriguing for further study (Chou and Wang, 2016; Dumpich et al., 2015; Irie and Yamaguchi, 2004; Kessels et al., 2011; Meyer and Feldman, 2002; Zallen et al., 2002).

In Drosophila, SCAR is particularly important for cytoplasmic organization in the blastoderm, for egg chamber structure during oogenesis, axon development in the central nervous system, and adult eye morphology (Zallen et al., 2002). To further characterize the relationship between Non-stop, Atxn7 and WRC, we turned to the BG3 cell line (ML-DmBG3-c2, RRID:CVCL_Z728). These cells were derived from the Drosophila third instar larval central nervous system. Biochemical and RNAseq profiling of these cells indicate they express genes associated with the central nervous system, express neurotransmitters, are specifically detected with reagents used to probe for neuronal cells (such as HRP), and readily form neuroblasts. Their morphology is similar to that of mammalian astrocytes, which are used widely as a model for neurodegenerative disease. (Cherbas et al., 2011; Ui-Tei et al., 1994; Ui-Tei et al., 1995; Ui et al., 1994).

To verify interaction between Non-stop and WRC, BG3 cells were transiently transfected with either Non-stop-FH or SCAR-2xFLAG-2xHA (SCAR-FH) and immunoprecipitated with anti-FLAG resin. An anti-HA antibody was used to verify pull down of the tagged protein. A previously verified anti-SCAR antibody confirmed the presence of SCAR in Non-stop-FH immunoprecipitate (Figure 2B) (Cetera et al., 2014; Evelyn Rodriguez-Mesa, 2012; Tran et al., 2015; Verboon and Parkhurst, 2015; Verboon et al., 2015). Reciprocal immunoprecipitation of SCAR-FH utilizing an anti-FLAG resin followed by immunoblotting for Non-stop using a previously characterized anti-Non-stop antibody confirmed endogenous Non-stop associated with SCAR-FH (Figure 2C) (Mohan et al., 2014b).

To visualize the scope of Non-stop and WRC colocalization, indirect immunofluorescence was used to localize endogenous SCAR and Non-stop in BG3 cells. In these stationary cells, SCAR localized throughout the cytoplasm, reaching into long bimodal processes extending from the cell body (Figure 2D). Non-stop localized similarly to SCAR, but also within nuclei. When we examined the spatial relationship between Non-stop and F-actin, using phalloidin, we observed Non-stop overlapping with portions of phalloidin (Figure 2E) (Cooper, 1987).

Atxn7 and Non-stop coordinate SCAR protein levels

The interaction of the DUBm with SAGA is regulated by Atxn7, meaning the amount of Non-stop available for interaction with SCAR increases upon loss of Atxn7 (Mohan et al., 2014b). If Non-stop was acting as a SCAR deubiquitinase to counter proteolytic degradation, then reducing Non-stop would lead to less SCAR protein. Conversely, we would expect increased SCAR protein upon loss of Atxn7. To test these hypotheses, we used RNAi to knock down Non-stop or Atxn7 transcripts in cell culture and observed SCAR protein levels by immunoblotting. BG3 cells were soaked in dsRNA targeting LacZ (negative control), Non-stop, or Atxn7. After five days, denaturing whole cell protein extracts were prepared from these cells and immunoblotted to detect SCAR. Ponceau S total protein stain was used as loading control to verify analysis of equal amounts of protein (Lee et al., 2016; Romero-Calvo et al., 2010). Protein extracts from Non-stop knockdown cells consistently showed about 70% decrease in SCAR protein levels relative to LacZ control knock-down. Consistent with a model in which Atxn7 restrains Non-stop deubiquitinase activity, cells treated with Atxn7-targeting RNAs showed 2.5-fold increase in SCAR protein (Figure 3A). We verified these results by immunoblotting whole cell extracts prepared from the brains of 3rd instar larvae bearing homozygous mutations for *non-stop (not^{02069})*, a loss of function allele (Poeck et al., 2001) or *Atxn7* (*Ataxin7$^{[KG02020]}$*), a loss of function allele (Mohan et al., 2014b). Larval brain extracts showed about 75% decrease in SCAR protein levels in *non-stop* mutants (Figure 3B). In *Atxn7* mutant extracts, SCAR was increased 2.5-fold (Figure 3B).

Considering that SAGA is a known chromatin modifying complex best characterized for its role in gene regulation. We examined whether Non-stop influences *SCAR* gene expression. In genome wide analysis of glial nuclei isolated from mutant larvae, *SCAR* transcript was significantly down regulated by one fold in Non-stop deficient glia, but not in *Sgf11* deficient glia (Ma et al., 2016). Since Sgf11 is required for Non-stop enzymatic activity, it is not clear what mechanism links Non-stop loss to reduction in *SCAR* gene expression. To confirm the

relationship between Non-stop and *SCAR* gene expression, we used qRT-PCR to measure *SCAR* transcript levels in BG3 cells knocked-down for *non-stop*. We found a similar one half decrease in *SCAR* transcript levels (Figure 3C). However, examination of *Atxn7* knockdown showed no decrease in *SCAR* transcript levels (Figure 3C). These results suggest *non-stop* loss leads to modest decreases in *SCAR* transcripts, but *SCAR* gene expression is independent of *Atxn7* or *Sgf11*, suggesting this gene is not SAGA-dependent or dependent on Non-stop enzymatic activity. To determine the consequences of reducing *SCAR* transcripts by half on the level of SCAR protein, we examined *SCAR* heterozygous mutant larval brains ($SCAR^{[Delta37]}$/+). qRT-PCR analysis showed heterozygous *SCAR* brains produced 40% less *SCAR* transcript than wild-type brains (Figure 3D). However, heterozygous *SCAR* mutant and wild-type brains showed similar amounts of protein, demonstrating this amount of transcript is sufficient for full production of SCAR protein (Figure 3E). Since *non-stop* mutants showed a similarly modest decrease in *SCAR* gene expression but a contrasting drastic decrease in protein levels, and considering the interaction of DUBm and WRC proteins, we tested the possibility that Non-stop counters SCAR protein degradation post-translationally.

Purification and mass spectrometry identified WRC members SCAR, HEM, and SRA-1, as interaction partners of the independent DUBm (Figure 2A). In Drosophila, HEM, SRA-1, and Abi recruit an unidentified deubiquitinase to balance SCAR regulation through a constant ubiquitination-proteasomal degradation mechanism facilitating rapid changes in SCAR protein amount and localization (Kunda et al., 2003). In mammals, the USP7 deubiquitinase acts as a molecular rheostat controlling proteasomal degradation of WASH (Hao et al.). To determine whether Non-stop similarly regulates SCAR entry into a proteasomal degradation pathway in Drosophila we utilized *ex vivo* culture of 3rd instar larval brains to permit pharmacological inhibition of the proteasome (Rabinovich et al., 2015; Ranjini Prithviraj, 2012). Wandering 3rd instar larval brains were isolated from *non-stop* homozygous mutant larvae and cultured for 24 hours in the presence of MG132 proteasome inhibitor. Treating *non-stop* brains with MG132 rescued SCAR protein amounts to levels found in wild type brains (Figure 3F). Rescue of SCAR protein by proteasome inhibition suggests Non-stop counteracts SCAR protein entry into a proteasomal degradation pathway.

Non-stop regulates SCAR protein levels and localization

To examine whether increasing Non-stop led to increased SCAR, we expressed recombinant Non-stop in BG3 cells and utilized indirect immunofluorescence to observe the amount and location of endogenous SCAR protein. Upon expression of Non-stop-FH in BG3

cells, endogenous SCAR protein levels increased 5-fold compared with mock transfected cells (Figure 4A). Utilizing anti-HA immunofluorescence to observe recombinant Non-stop, three distinct subcellular localization patterns were apparent: nuclear, cytoplasmic, and evenly distributed between nucleus and cytoplasm (Figure 4A). Increased SCAR protein co-compartmentalized with Non-stop, even within the nucleus, where SCAR function is more enigmatic (Figure 4A). To quantify co-compartmentalization of SCAR and Non-stop, we calculated the nuclear to cytoplasmic ratios of Non-stop-FH and endogenous SCAR by dividing immunofluorescence intensity within each nuclei by that of the corresponding cytoplasm. This was plotted as the ratio nuclear:cytoplasmic SCAR on the Y axis and the ratio nuclear:cytoplasmic Non-stop on the X axis. As the amount of Non-stop in the nucleus increased, so did the amount of SCAR in the nucleus, showing that Non-stop was driving SCAR localization (R^2=0.8) (Figure 4A).

SCAR functions, within the WRC, to activate Arp2/3 and facilitate branching of F-actin filaments (Kurisu and Takenawa, 2009). With correct spatiotemporal regulation of these actin-branching complexes, cells are able to establish appropriate shape and size through the extension of actin-based protrusions (Blanchoin et al., 2014). If Non-stop overexpression were disrupting SCAR function through augmenting and mislocalizing SCAR protein, we expected to observe changes in cell shape and ability to form protrusions. When we used phalloidin to observe F-actin in cells overexpressing Non-stop, a substantial change in cell morphology was observed. Cells displayed fewer projections and covered less total area (Figure 4B). This was especially pronounced in cells expressing Non-stop in the cytoplasm or both nucleus and cytoplasm. Overexpression of SCAR alone led to similar reductions in cell area and number of projections observed. Increased Non-stop signal associated closely with increased phalloidin signal.

Non-stop bears multiple, conserved, WRC interacting receptor sequence (WIRS) motifs

We sought the basis for interaction between the SAGA DUBm, Arp2/3, and WRC. Some SCAR-interacting proteins bear a WRC interacting receptor sequences: (WIRS) motif (Chen et al., 2014). Primary sequence analysis of DUBm subunits revealed four WIRS-conforming sequences on Non-stop and the mammalian orthologue USP22. These were conserved in number and relative location (Figure 5A). With the exception of Non-stop, none of the DUBm components bore conserved WIRS motifs. Therefore, we focused on Non-stop WIRS motifs. These were numbered from 0 to 3, with WIRS1, 2, and 3 conserved in location between Drosophila and mammalian Non-stop (Figure 5A). Analysis of the yeast orthologue of Non-stop

(UBP8) did not reveal a WIRS-like motif, but the Atxn7 orthologue Sgf73 did. In yeast, the DUBm is also be found separate from SAGA, although not without Sgf73 (Lim et al., 2013).

To determine if Non-stop WIRS sequences are relevant for functional interaction with SCAR, we produced a series of phenylalanine to alanine point mutants in the putative WIRS domains (WIRS F-A) and expressed them in BG3 cells. As above, we measured the ability of Non-stop WIRS mutants to increase the levels of endogenous SCAR protein, to change cell area, and to change the number of protrusions per cell. Immunofluorescence was used to detect the HA epitope on exogenously expressed Non-stop and an antibody toward endogenous SCAR was used to observe SCAR protein levels. We analyzed cells expressing moderate amounts of Non-stop as judged by the spectrum of fluorescence intensity to avoid analyzing cells with either too little or too much exogenous Non-stop relative to endogenous protein. We calculated the ratio of HA fluorescence to SCAR fluorescence in each mutant and compared this to wild-type Non-stop (Figure 5B). In each case, the mutant Non-stop produced less SCAR protein than wild type, suggesting that these motifs are indeed important for Non-stop mediated increases in SCAR protein levels (Figure 5B). Mutant Non-stop was also less potent for decreasing cell area and decreasing the number of cell protrusions (Figure 5C).

Discussion

Purification of Atxn7-containing complexes indicated that Atxn7 functions predominantly as a member of SAGA (Mohan et al., 2014b). In yeast, the Atxn7 orthologue, Sgf73, can be separated from SAGA along with the deubiquitinase module by the proteasome regulatory particle (Lim et al., 2013). Without Sgf73, the yeast deubiquitinase module is inactive (Samara et al., 2010b). In higher eukaryotes Atxn7 increases, but is not necessary for Non-stop/USP22 enzymatic activity in vitro (Lan et al., 2015; Mohan et al., 2014b). In Drosophila, loss of Atxn7 leads to a Non-stop overactivity phenotype, with reduced levels of ubiquitinated H2B observed (Mohan et al., 2014b). Purification of Non-stop revealed the active SAGA DUBm associates, separate from SAGA, with multi-protein complexes including WRC and Arp2/3 complexes. SCAR was previously described to be regulated by a constant ubiquitination/deubiquitination mechanism (Kunda et al., 2003). We found increased SCAR protein levels upon knockdown of *Atxn7* and decreased SCAR protein levels upon knockdown of *non-stop*. We found that that decreases in SCAR protein levels in the absence of *non-stop* required a functional proteasome.

When we overexpressed Non-stop, we found an increase in SCAR protein levels. Increases in SCAR protein colocalized to subcellular compartments where Non-stop protein levels increased. Nuclear Arp2/3 and WRC have been linked to nuclear reprogramming during

early development, immune system function, and general regulation of gene expression (Miyamoto et al., 2013; Taylor et al., 2010; Yoo et al., 2007). Distortions of nuclear shape have been shown to alter chromatin domain location within the nucleus, resulting in changes in gene expression (Evelyn Rodriguez-Mesa, 2012; Navarro-Lerida et al., 2015).

When we examined the basis for this unexpected regulatory mechanism, we uncovered a series of WIRS motifs (Chen et al., 2014) conserved in number and distribution between flies and mammals. These sequences functionally modulate Non-stop ability to increase SCAR protein levels. Point mutants of each WIRS resulted in less SCAR protein per increase in Non-stop protein.

Overall, these findings suggest that SAGA maintains a pool of Non-stop that can be made available to act distally from the larger SAGA complex to modulate SCAR protein levels (Figure 6). Already, it has been shown that in yeast, the proteasome regulatory particle can mediate removal of the DUBm from SAGA (Lim et al., 2013). In higher eukaryotes, caspase 7 cleavage has been shown to act on Atxn7 at residues which would be expected to release the DUBm, although this has not been explicitly shown (Young et al., 2007). The mechanisms orchestrating entry and exit of the DUBm from SAGA remain to be explored.

Figure Legends

Figure 1. Atxn7 co-purifies with the Spt Ada Gcn5 acetyltransferase (SAGA) complex. Non-stop (Not) co-purifies with SAGA and additional multi-protein complexes. (A) Working model: The Non-stop-containing deubiquitinase module (DUBm) functions distally from SAGA. Non-stop is anchored to SAGA by Atxn7 but is released to interact with unknown partners. (B) Scheme to identify Non-stop interactors. (C) Protein complexes, purified and fractionated as described in B were subject to the ubiquitin-AMC assay to measure deubiquitinase activity where fluorescence increases as deubiquitination occurs. Top panel is the measure of activity when Atxn7 is used as bait. Bottom panel is the measure of activity when Non-stop is bait. (D) Fractions from Non-stop-containing complexes were split into three groups, indicated in lower panel of C, and constituent proteins identified by mass spectrometry. Distributed normalized spectral abundance factor (dNSAF) values for SAGA complex members revealed Spt (NippedA) and KAT (Ada2b) modules of SAGA were only found in group 1 (SAGA). DUBm subunits (Non-stop and Sgf11), however, were found in groups 1 and 2 (Other multi-protein complexes).

Figure 2. WAVE regulatory complex (WRC) and Arp2/3 interact with the SAGA DUBm. (A) Mass spectrometry analysis of Group 2 fractions revealed that Arp2/3 and WRC complexes stably interact with Non-stop. “Total” represents purified complexes applied to the Superose 6 gel filtration column. None of these proteins were identified using vector only control purifications. (B and C) Reciprocal pull-down verifies interaction between Non-stop and WRC subunit SCAR. (B) BG3 cells were transfected with an expression vector for Non-stop-2xFLAG-2xHA (Non-stop-FH) or no vector (Control). Recombinant Non-stop was immunoprecipitated using anti-FLAG affinity resin and purified interactors analyzed by immunoblotting for the presence of endogenous SCAR. Anti-HA immunoblots verified the presence of Non-stop-FH bait. NS marks a non-specific band. (C) Pull-down was performed as in B, but with SCAR-2xFLAG-2xHA (SCAR-FH) expression vector. Immunoprecipitated proteins were probed by immunoblotting to detect endogenous Non-stop. Anti-HA immunoblots verified the presence of SCAR-FH bait. (D) Non-stop and SCAR occupy similar subcellular regions. Immunofluorescence of SCAR and Non-stop in BG3 cells. Cells were immunostained with anti-Non-stop (magenta), anti-SCAR (yellow), and DAPI (white). Scale bar is 10 µM. Inset is enlarged to the right. (E) Non-stop and F-actin are found in similar subcellular regions. Representative images of BG3 cells immunostained with anti-Non-stop (magenta), phalloidin (F-actin)(yellow), and DAPI (DNA)(white). Scale bar is 10 µM. Inset is enlarged to the right.

Figure 3. Non-stop and Atxn7 regulate SCAR protein levels. (A) BG3 cells were treated with dsRNA to knock down *Atxn7*, *LacZ* (control), or *non-stop* (*not*). Denaturing whole cell extracts were analyzed by western blotting to determine SCAR protein levels. Total protein (Ponceau, lower panel) was used as loading control. Three biological replicates were analyzed by immunoblotting and SCAR intensity quantified by densitometry. Values were normalized to Ponceau and expressed relative to *LacZ* control (right panel). (B) Brain and central nervous systems from third instar larvae, mutant for the indicated genes, were isolated and denaturing whole cell extracts prepared. SCAR protein levels were determined by immunoblotting and quantified as above. Wild-type (WT) is OregonR, *not* is *non-stop*^{02069}, and *Atxn7* is *Ataxin7*$^{[KG02020}$. (C) Relative quantification of SCAR transcripts in BG3 cells treated with dsRNA targeting either *Atxn7*, *LacZ* (control), or *not*. Transcript levels from *LacZ* treated cells were set as one and transcripts from *not* and *Atxn7* treated cells are expressed relative to *LacZ*. (D) Relative quantification of *SCAR* transcript levels in larval brains dissected from WT or *SCAR*$^{[Delta37]}$/cyo-gfp (*SCAR*/+). *SCAR* transcript levels are set relative to WT. (E) Immunoblot for SCAR protein in larval brains dissected from OregonR (WT) or *SCAR*$^{[Delta37]}$/cyo-gfp (*SCAR*/+). (F) WT or *not* brains were dissected from 3rd instar larva and cultured *ex vivo*. Half of the brains from each genotype were treated for 24 hours with protease inhibitor [50 µm MG132 (MG132+)] while the remaining brains served as untreated control (MG132-). Whole cell extracts were analyzed as above to quantify SCAR protein amounts.

Figure 4. Overexpression of Non-stop increases SCAR levels, directs SCAR localization, and alters cell morphology – reducing cell area and number of cell protrusions. (A) Augmenting Non-stop increases local levels of SCAR. Drosophila BG3 central nervous system cells were transfected with no plasmid (control) or with Non-stop-FH expression vector as indicated. Exogenous Non-stop and endogenous SCAR were located and quantified through nuclear localization by DAPI (DNA) (white) and indirect immunofluorescence toward: Non-stop-HF (anti-HA) (magenta), anti-SCAR (yellow). Scale bar is 10 µM. Hashed lines outline DAPI (inner circle) to approximate nuclear location and the outermost SCAR signal to approximate the cell edge (outer circles). Three categories of HA (Non-stop-FH) expression are shown: Non-stop ‘nuclear’, Non-stop ‘cytoplasmic’, and Non-stop ‘nuclear and cytoplasmic.’ Total SCAR signal was measured in HA positive cells (Non-stop-FH) and mock transfected cells (Control). The average increase in endogenous SCAR upon expression of exogenous Non-stop was determined relative to control (top, right panel). To examine the relationship between Non-stop localization and increased SCAR protein, SCAR immunofluorescence intensity was measured in the

nucleus (as defined by DAPI) and in the cytoplasm. A ratio of nuclear SCAR intensity divided by cytoplasmic SCAR intensity was calculated. HA intensity was similarly measured in the nucleus and cytoplasm. A ratio of nuclear HA intensity to cytoplasmic HA intensity was calculated. The HA ratio was plotted on the X axis and the SCAR ratio was plotted on the Y axis. A trend line was calculated and the R^2 was determined to be 0.80. (B) Increasing Non-stop alters F-actin organization similarly to increasing SCAR – reducing cell area and number of cell protrusions. BG3 cells were transfected with no-plasmid (Control), Non-stop-FH, or SCAR-FH. The location of exogenously expressed proteins were determined as above and F-actin was visualized using phalloidin. Cell area and total number of cellular projections were counted by quantifying the phalloidin signal distribution. HA-positive cells (Non-stop-FH or SCAR-FH) were compared to mock transfected (Control) cells (bottom panels). Cells expressing Non-stop-FH were split into three categories of HA (non-stop-FH) expression as above. T-tests were used to compare samples and p-values are shown.

Figure 5. Non-stop bears conserved WIRS motifs. Mutating these reduces Non-stop ability to increase SCAR levels, reduce cell area, and reduce number of cell protrusions. (A) Alignment of Non-stop protein sequences reveals a conserved distribution of WIRS motifs between higher eukaryotes. (B) BG3 cells were transfected with either wild type (WT) or Non-stop-FH harboring a point mutation in one of the four putative WIRS motifs (W0-W3) as indicated. Exogenously expressed Non-stop and endogenous SCAR were detected by indirect immunofluorescence as above. Total fluorescence intensity of SCAR and HA were measured in HA containing cells. Box plots show the SCAR intensity divided by the intensity of HA. T-tests were used to compare samples and p-values are shown. (C) BG3 cells transfected with either wild-type or Non-stop-FH harboring a point mutation in one of the four putative WIRS motifs (W0-W3) were immunostained for HA (magenta) and phalloidin (yellow). Scale bar is 10 µm. Arrows point to localized accumulation of Non-stop WIRS mutant protein, which coincide with aberrant actin protrusions commonly observed upon WRC or Arp2/3 loss of function. Cell area and number of projections protruding from cells was determined for HA positive cells. T-tests were used to compare the samples and p-values are shown.

Figure 6. Non-stop regulates SCAR protein levels and location. Model showing Non-stop interacts with WRC in an Atxn7-dependent manner, where Non-stop then counteracts degradation of WRC subunit SCAR. Loss of Atxn7 leads to increased availability of Non-stop for interaction with WRC.

Methods

Cell culture

BG3 cells were grown in Schneider's media supplemented with 10% fetal bovine serum and 1:10,000 insulin. S2 cells were maintained in Schneider's media supplemented with 10% fetal bovine serum and 1% pen-strep.

Transfection

BG3 cells were plated at 0.5 x10^6 cells/ml, forty eight hours later they were transfected using Lipofectamine 3000 (ThermoFisher catalog number: L3000015) following the manufacturer's directions. The Lipofectamine 3000 was used at 1 µl per µg of DNA and p3000 reagent was used at 2 µl per µg of DNA to be transfected. To induce expression of proteins from plasmids containing a metalothienine promoter 250 µm of $CuSO_4$ was added to the media. For transfections taking place in 6 well dishes 1 µg of DNA was used per well and 15 µg of DNA was used per 15 cm dish. Proteins were given 2 days of expression before the samples were used.

Plasmids

Scar (Berkley Expression Clone Collection FMO14142) (Yu et al., 2011)
Prmha3-non-stop-2XFlag-2XHA (ubiquitin-specific protease nonstop [Drosophila melanogaster] Sequence ID: AAD53181.1) (Poeck et al., 2001)
Prmha3-non-stop-2XFlag-2XHA-0FA, Prmha3-non-stop-2XFlag-2XHA-1FA, Prmha3-non-stop-2XFlag-2XHA-2FA, Prmha3-non-stop-2XFlag-2XHA-3FA, and Prmha3-non-stop-2XFlag-2XHA-4FA were created using quick change mutagenesis using Prmha3-non-stop-2XFlag-2XHA as a template following the manufacturer's directions.
Primer list for quickchange mutagenisis:
WIRS0 phenylalanine to alanine F: GCAGTGGCCGAAGCGCCGGCAGGGGAACGGAACGGTGGGC
WIRS0 phenylalanine to alanine R: CCGTTCCCCTGCCGGCGCTTCGGCCACTGCTGCTGCTGC
WIRS1 phenylalanine to alanine F: CAGCTACGATACAGCCCGGGTCATCGACGCCTACTTCGCTGCTTGCG
WIRS1 phenylalanine to alanine R: GGCGTCGATGACCCGGGCTGTATCGTAGCTGTGCTCCTTCACATAGC
WIRS2 phenylalanine to alanine F: GTCTCTTCCAGGAGGCCTATTCCGGCTCCCGTTCGCCGCTGTCGC
WIRS2 phenylalanine to alanine R: GGAGCCGGAATAGGCCTCCTGGAAGAGACGTGACACTTCACAAACG
WIRS3 phenylalanine to alanine F: CCAGCGTGGTGTCGGCCCATTTGAAACGCTTCGAGCACTCAGCTCTG
WIRS3 phenylalanine to alanine R: CGAAGCGTTTCAAATGGGCCGACACCACGCTGGGCAGAGTGCGCAG
WIRS4 phenylalanine to alanine F: CGCAAGATCTCCTCGGCCATTCAATTCCCCGTGGAGTTCGACATG
WIRS4 phenylalanine to alanine R: CCACGGGGAATTGAATGGCCGAGGAGATCTTGCGATCGATCAGAGC

RNAi treatment of BG3 cells

Short dsRNA was produced by amplifying cDNA with primers that contained T7 promoter sequences on the ends. These PCR products were then used as a template for in vitro transcription using the Ampliscribe T7 high yield transcription kit (epicentre catalog number: AS3107) following the manufacturer's instructions. Transcription was allowed to continue for four hours. To make the RNA double stranded it was then heated at 65 °C for thirty minutes and cooled to room temperature. The RNA was then ethanol precipitated in order to clean it.

BG3 cells were plated at $2x10^6$ cells/ml in 6 well dishes. Cells were allowed to adhere (approximately four hours) then treated with 10 µg of dsRNA. The cells were harvested for western blot after being soaked for six days and after five days for RNA analysis.

RNAi

Atxn7 DRSC23138, Atxn7 DRSC35628, non-stop BKN21994, non-stop DRSC11378
dsRNA-LacZ-R: GCTAATACGACTCACTATAGGCCAAACATGACCARGATTACGCCAAGCT
dsRNA-LacZ-F: GCTAATACGACTCACTATAGGCCAAACGTCCCATTCGCCATTCAGGC

Immunofluorescence

Cells were plated directly onto coverslips in 6 well dishes and allowed to adhere. BG3 cells were fixed in 4% methanol free formaldehyde after two days of growth. Cells were washed in 1xPBS and then either stored in 1xPBS for up to one week or immediately stained. Coverslips were washed with 1xPBS containing 0.5% Triton X-100 four times for 10 minutes. Coverslips were blocked in 5% BSA and 0.2% TWEEN 20 diluted in 1xPBS for one hour at room temperature. They were then incubated in primary antibody diluted in blocking buffer overnight at 4 °C. The following day they were washed with 1xPBS containing 0.5% Triton X-100 four times for ten minutes. They were then incubated in secondary antibody diluted 1:1000 in blocking buffer for four hours at room temperature. Cells were then washed four times ten minutes at room temperature in 1xPBS containing 5% Triton X-100. Coverslips were dried and mounted in Vectashield containing DAPI (Vector Labs catalog number: H-1200).

A Zeiss LSM-5 Pascal upright microscope and LSM software was used for imaging. Cells were imaged with a 40X objective aperture number 1.30 oil or a 63X objective aperture number XXX and Z-stacks were acquired using LSM software with a slice every 1 µm. Brains were imaged with a 20X objective aperture number 0.45 air and Z stacks were taken with slices every 4 µm. All images were taken at room temperature. All settings remained the same within an experiment.

Antibodies

Mouse anti SCAR (DSHB P1C1-SCAR) was used at a dilution of 1:100. Rat anti HA (Roche catalog number: 11867423001) was used at a dilution of 1:1000. Guinea pig anti non-stop (Mohan et al., 2014b) was used at a dilution of 1:500. Goat anti Rat IGG -568 (Invitrogen Catalog number: A-11077) was used at a dilution of 1:1000. Goat anti Mouse IGG-488 (Invitrogen Catalog number: A-11001) was used at a dilution of 1:1000. Goat anti Guinea pig IGG- 488 (Invitrogen Catalog number: A11073) was used at a dilution of 1:1000. Phalloidin-488 (Invitrogen Catalog number: A12379) was used at a dilution of 1:20. Phalloidin-568 (Invitrogen Catalog number: A12380) was used at a dilution of 1:20.

Image analysis

Stacks were exported as TIFFs from the LSM software and analyzed in ImageJ. For analysis z projections were created by sum.

To analyze cell shape and phalloidin structures in BG3 cells the Phalloidin images were thresholded in ImageJ and used to create ROIs. The number of projections was counted by

hand. A t-test was used to test the significance between the mock transfected control and the non-stop-2xFlag-2 transfected cells lines in cellular shape and the number of projections.

To measure SCAR levels in BG3 and S2 cells ROIs were drawn by hand around the entirety of the scar staining and integrated density was measured for both HA and SCAR. For BG3 cells a second ROI was drawn around the nuclei and the scar intensity of the SCAR and HA was measured. Thresholding the original image and using image calculator subtracted background. A t-test was used to compare between treated and untreated cells.

Ex vivo culturing of third instar brains

Ex vivo culturing of larval brains was performed as described (Rabinovich et al., 2015; Ranjini Prithviraj, 2012). Third instar brains were dissected in 1xPBS and quickly placed into culture media (Schneider's insect media supplemented with 10% FBS, 1% pen/strp, 1:10,000 insulin, and 2 µg/ml ecdysone) in 24 well dish. The dish was wrapped in parafilm and incubated at 25 °C. After 6 hours of culturing the media was changed. The wells receiving MG132 then had 50 µM of MG132 added. Brains were collected for protein samples after 24 hours.

Protein Sample Preparation

All western blots using tissue culture cells were done from cells collected from a 6 well dish. The media was removed and 200 µl of 2xUREA sample buffer (50 mM Tris HCl pH 6.8, 1.6% SDS, 7% glycerol, 8M Urea, 4% β mercaptoethanol, 0.016% bromophenol blue) and 2 µl of 10 µg/ml benzonase was added to the dish. After the mixture was lysed they were boiled at 80 °C for five minutes.

Larval brains were dissected into 1xPBS. The PBS was removed and 2xUREA sample buffer was added in a 1:1 ratio and 10 units of Benzonase was added. The brains were homogenized by pipetting and boiled at 80 °C for five minutes.

Immunoprecipitation

Transfected cells were resuspended in Extraction Buffer (20 mM HEPES (pH7.5), 25% Glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1:100 ethidium bromide. 1% NP-40 was added and the cells were pipetted up and down until the solution was homogenous. They were placed on ice for one hour with agitation every 10-15 minutes. They were then centrifuged for 45 minutes at 4 °C at 4,500 x g. The supernatant was placed into a new tube and stored at – 80 °C for up to one week. Lysates were thawed and 36 µg of protein was removed for input and mixed with 2 x Urea sample buffer (50 mM Tris-HCl (pH 6.8), 1% SDS, 7% Glycerol, 8 M Urea, 4% beta-mercapto-ethanol, 0.016% Bromophenol blue) and boiled for five minutes at 80 °C. An equal volume of dignum buffer (10 mM HEPES (pH 7.5), 1.5 mM $MgCl_2$, 10 mM KCl) was added to the lysates in order to adjust the salt concentration to 210 mM NaCl. The entire volume was added to 15 µl of Mouse IGG agarose (Sigma Catalog number: A0919) that had been equilibrated in one volume extraction buffer plus one volume dignum. They were then rotated at 4 °C for one hour. After one hour they were centrifuged for one minute at 4 °C at 4,500 x g. The supernatant was then added to anti-FLAG M2 Affinity Gel (Sigma Catalog number: A2220) that had been equilibrated in one volume extraction buffer plus one volume dygnum. They were then rotated for one hour at 4 °C. After one hour they were centrifuged at 4,500 x g at 4 °C for thirty seconds. The supernatant was removed and the gel was washed five times in extraction buffer

plus dygnum. The resin was then resuspended in 40 µl 2XUREA sample buffer and boiled for five minutes at 80 °C.

Western Blot

Protein samples were run on either 6% polyacrylamide gel (ImmunoPreciptiaton) or on 8% polyacrylamide gels (all other samples) in 1X Laemli electrode buffer. Proteins were transferred to Amersham Hybond P 0.45 µM PVDF membrane (catalog number: 10600023) using the Trans-blot turbo transfer system from biorad. The semidry transfer took place in Bjerrum Schafer-Nielsen Buffer with SDS (48 mM Tris, 39 mM Glycine, 20% Methanol, 0.1% SDS) at 1 Amp 25 V for 20 minutes. Membranes were stained in Ponceau (0.2% Ponceau S, 2 % Acetic Acid) for twenty minutes at room temperature as a loading control. The membrane was then briefly washed in 1XPBS and imaged on a Chemidoc MP Imaging system. Membranes were then washed three times for five minutes to remove excess ponceau. Membranes were then blocked for one hour in 5% non-fat dried milk diluted in 0.05% Triton-X 100 in 1XPBS. The membrane was then briefly rinse and incubated in primary antibody diluted in 1% non-fat dried milk diluted in 0.05% Triton-X 100 for either one hour at room temperature or overnight at 4°C. The membrane was then washed four times for five minutes in 1% Triton-X 100 diluted in 1XPBS. The membrane was then incubated for one hour at room temperature in secondary antibody diluted 1:5000 in 1% non-fat dried milk diluted in 0.05% Triton-X 100. The membrane was then washed four times for five minutes in 1% Triton-X 100 diluted in 1xPBS. Bioworld ICL (catalog number: 20810000-1) was used to visualize the proteins following the manufacturer's instruction. The chemiluminescence was imaged on a Chemidoc MP Imaging system. Band intensities were measured on ImageLab software.

Antibodies

Mouse anti SCAR (DSHB P1C1-SCAR) was used at a dilution 1:500. Rat anti HA-HRP (Roche catalog number: 12013819001) was used at a dilution of 1:1000. Guinea Pig anti non-stop (Mohan et al., 2014b) was used at a dilution of 1:1000. All secondary anti-bodies were used at 1:5000: Goat anti Guinea Pig HRP (Jackson ImmunoResearch INC catalog number: 106-035-003) and Goat anti mouse HRP (Jackson ImmunoResearch INC catalog number: 106-035-003).

QPCR

RNA was extracted using the Invitrogen PureLink RNA mini kit (Fisher catalog number: 12 183 018A) following the manufacturer's instructions. The optional on column DNA digest was performed using the PureLink DNase set (Fisher Catalog number: 12185010). The high capacity cDNA reverse transcription kit (ThermoFisher catalog number: 4374966) was used to create cDNA using the manufacturer's protocol. 3 µl of cDNA created from 1 µg of RNA was used in downstream qPCR analysis. TaqMan gene expression assays were run following the manufacturer's directions. PCR was performed using the TaqMan Universal PCR Master Mix (ThermoFisher catalog number: 4364340). The reactions were run on an ABI 7500 Real-time PCR machine.

TaqMan assay IDs

RpL32 Dm02151827_g1, SCAR Dm01810606_g1, Not Dm01823071_g1, Atxn7 Dm01800874_g1

Fly Strains

SCAR [Delta37]: w[*]; SCAR[Delta37] P(ry[+t7.2]=neoFRT)40A/CyO, , P(w[+mC]=GAL4-twi.G)2.2, P(UAS-2xEGFP)AH2.2. Atxn7: y[1] w[67c23]; P(y[+mDint2] w[BR.E.BR]=SUPor-P)CG9866[KG02020]/CyO, P(w[+mC]=GAL4-twi.G)2.2, P(UAS-2xEGFP)AH2.2. non-stop: P(ry[+t7.2]=PZ)not[02069] ry[506]/TM3, P(w[+mC]=GAL4-twi.G)2.3, P(UAS-2xEGFP)AH2.3, Sb[1] Ser[1]. Wild type: Oregon-R

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Figure 1

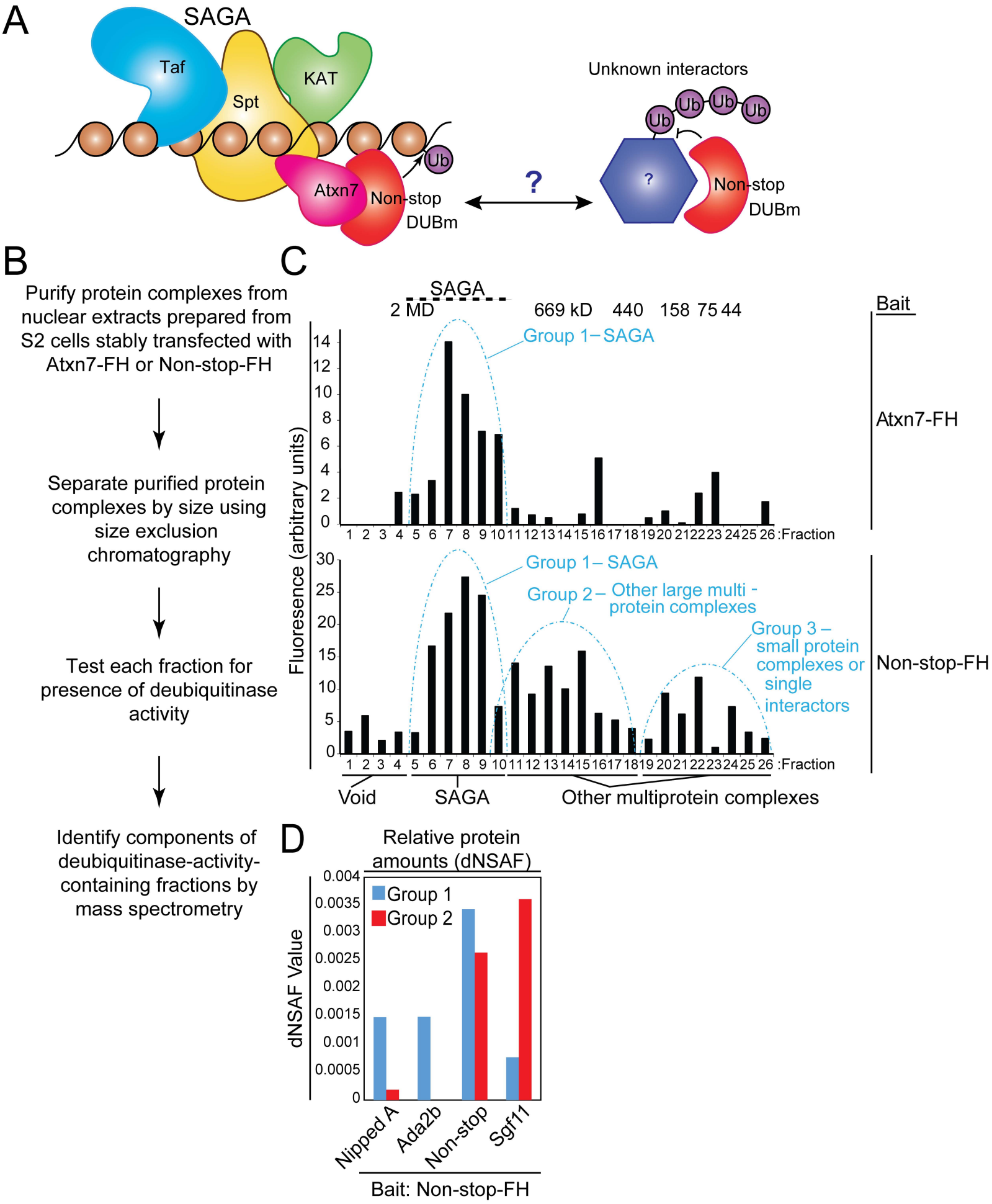
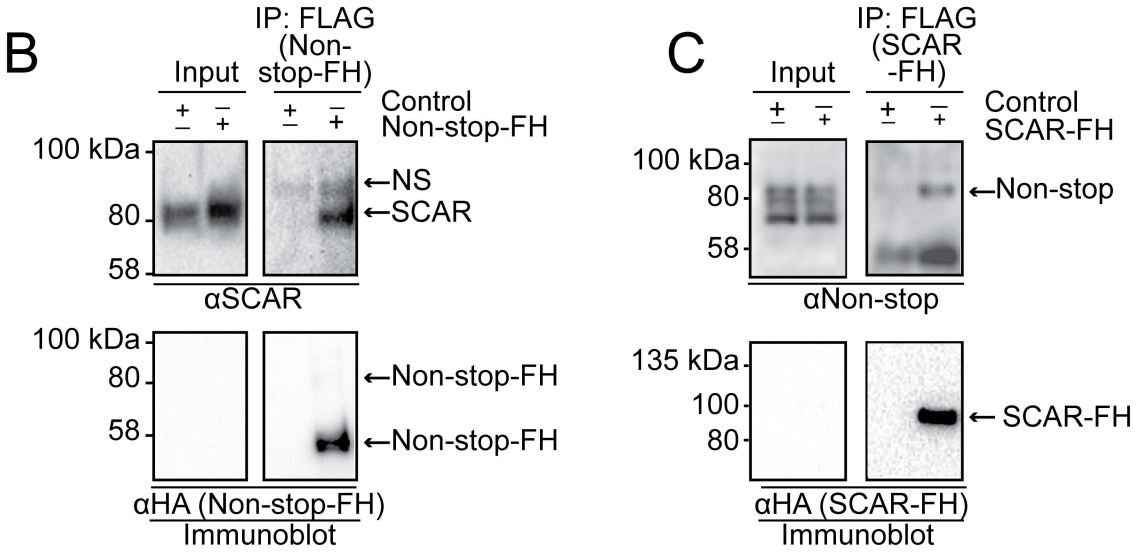

A
SAGA
Taf
Spt
KAT
Atxn7
Non-stop
DUBm
Ub
?
Unknown interactors
Ub
Ub
Ub
Ub
?
Non-stop
DUBm
B
Purify protein complexes from nuclear extracts prepared from S2 cells stably transfected with Atxn7-FH or Non-stop-FH
Separate purified protein complexes by size using size exclusion chromatography
Test each fraction for presence of deubiquitinase activity
Identify components of deubiquitinase-activity-containing fractions by mass spectrometry
C
SAGA
2 MD
669 kD
440
158
75
44
Bait
Group 1– SAGA
Atxn7-FH
Fluoresence (arbitrary units)
:Fraction
Group 1– SAGA
Group 2 – Other large multi - protein complexes
Group 3 – small protein complexes or single interactors
Non-stop-FH
Void
SAGA
Other multiprotein complexes
D
Relative protein amounts (dNSAF)
Group 1
Group 2
dNSAF Value
Nipped A
Ada2b
Non-stop
Sgf11
Bait: Non-stop-FH

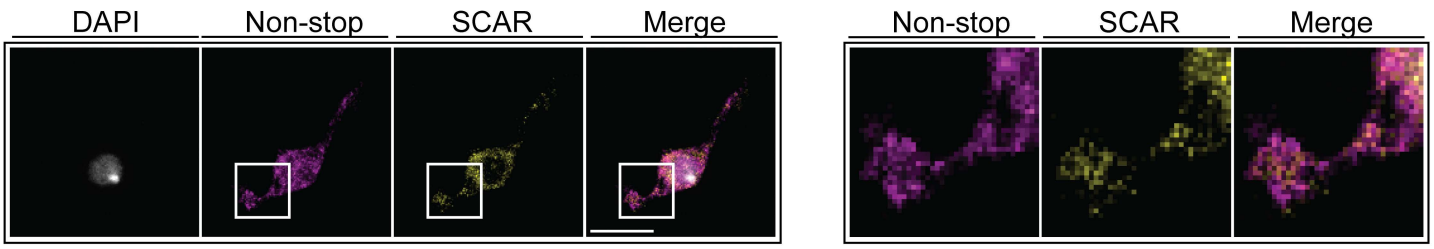
Figure 2

A

Protein name		Total input				Non-stop "Group 2" fraction				Vector only control		
Description	NCBI_Gene	Spectra	Sequence coverage	dNSAF		Spectra	Sequence coverage	dNSAF		Spectra	Sequence coverage	dNSAF
Sgf11	Sgf11	7	12.2	8.1E-4		2	8.7	7.7E-4		0	0	0
Enhancer of yellow 2	e(y)2	19	17.8	4.3E-3		15	25.7	1.1E-2		0	0	0
Non-stop	not	51	20.2	1.6E-3		32	15.7	3.4E-3		0	0	0
histone H2A	His2A	75	25.8	1.4E-2		40	31.5	2.4E-2		0	0	0
histone H2B	His2B	30	26.8	5.5E-3		1	7.3	6.1E-4		0	0	0
Actin-related protein 3	Arp3	39	34.7	2.1E-3		20	31.1	3.6E-3		0	0	0
Arpc3A, isoform C	Arpc3A	31	11.9	4.0E-3		14	21.5	6.0E-3		0	0	0
Arpc2	Arpc2	37	16.0	2.8E-3		5	12.3	1.3E-3		0	0	0
Arpc4	Arpc4	5	18.5	6.8E-4		7	19.1	3.1E-3		0	0	0
Arpc3B, isoform B	Arpc3b	1	8.9	1.4E-4		1	13.4	4.8E-4		0	0	0
Actin-related protein 2	Arp2	3	3.65	1.7E-4		4	3.6	7.7E-3		0	0	0
suppressor of profilin 2	Sop2	3	7.2	1.8E-4		9	14.3	1.8E-3		0	0	0
Arpc5	Arpc5	13	37.8	2.0E-3		7	24.5	3.5E-3		0	0	0
SCAR	SCAR	0	0	0		2	2.2	2.5E-4		0	0	0
HEM-protein	Hem	2	1.1	4.0E-5		1	1.1	4.3E-5		0	0	0
SRA-1	Sra-1	2	1.1	1.2E-4		4	2.2	1.5E-4		0	0	0



D



E

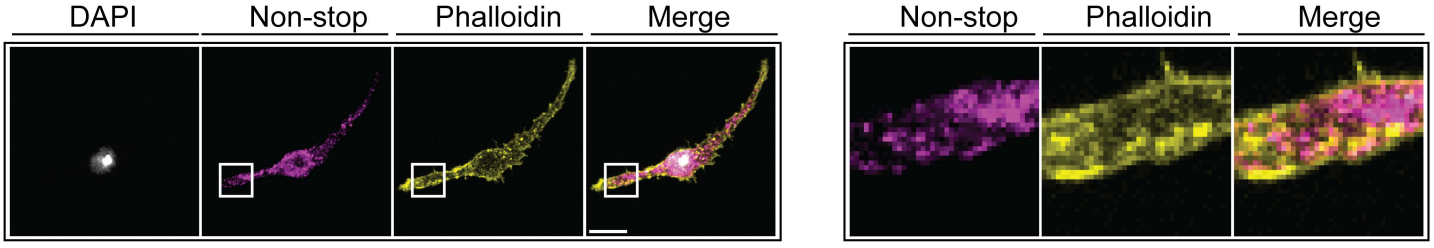


Figure 3

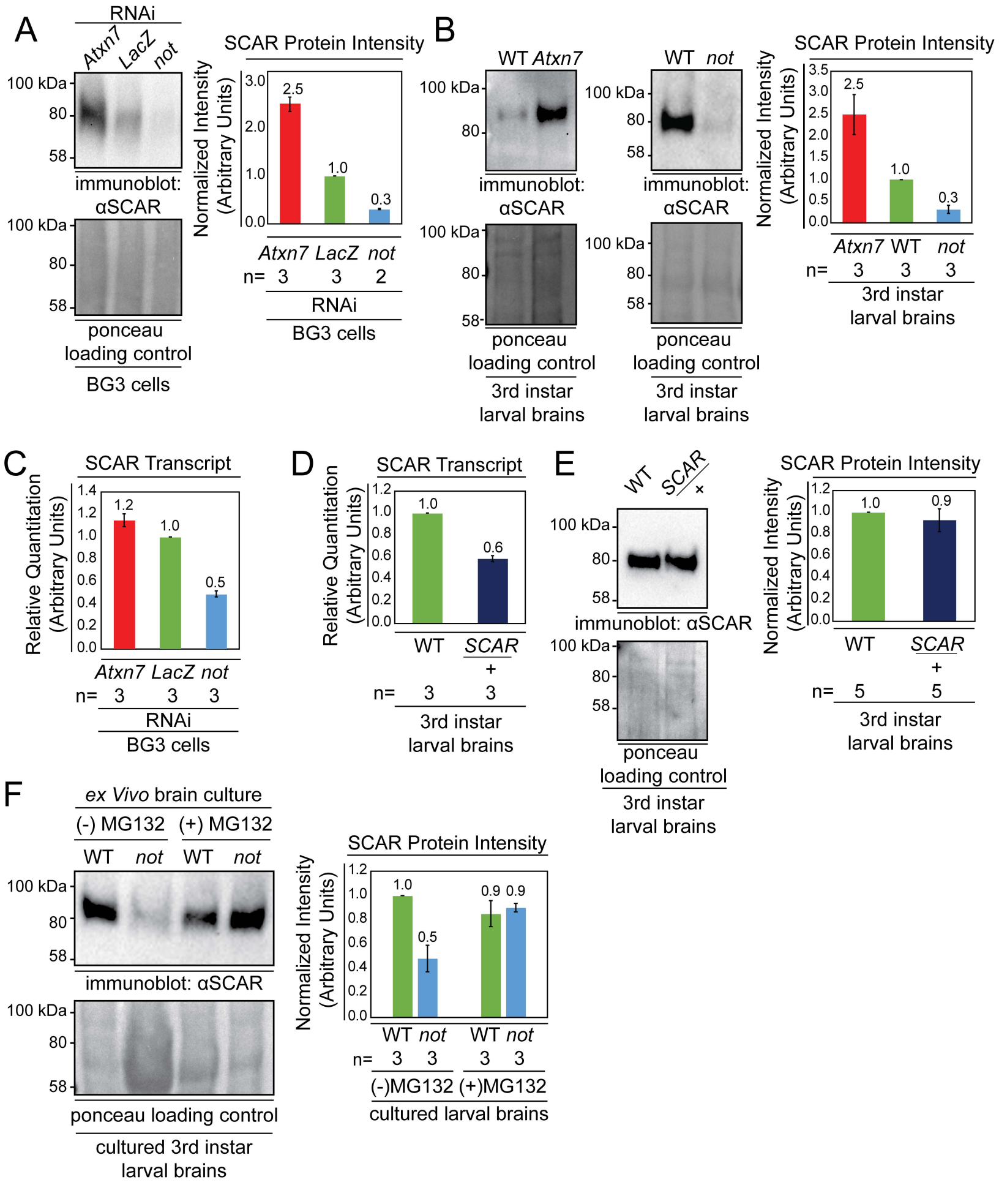

A
RNAi
Atxn7
LacZ
not
100 kDa
80
58
immunoblot: αSCAR
ponceau loading control
BG3 cells
SCAR Protein Intensity
Normalized Intensity (Arbitrary Units)
2.5
1.0
0.3
Atxn7 LacZ not
n= 3 3 2
RNAi
BG3 cells
B
WT Atxn7
WT not
immunoblot: αSCAR
ponceau loading control
3rd instar larval brains
SCAR Protein Intensity
Atxn7 WT not
n= 3 3 3
C
SCAR Transcript
Relative Quantitation (Arbitrary Units)
1.2
1.0
0.5
Atxn7 LacZ not
n= 3 3 3
RNAi
BG3 cells
D
SCAR Transcript
1.0
0.6
WT SCAR +
n= 3 3
3rd instar larval brains
E
WT
SCAR +
immunoblot: αSCAR
ponceau loading control
3rd instar larval brains
SCAR Protein Intensity
1.0
0.9
WT SCAR +
n= 5 5
3rd instar larval brains
F
ex Vivo brain culture
(-) MG132
(+) MG132
WT not WT not
immunoblot: αSCAR
ponceau loading control
cultured 3rd instar larval brains
SCAR Protein Intensity
1.0
0.5
0.9 0.9
WT not WT not
n= 3 3 3 3
(-)MG132 (+)MG132
cultured larval brains

Figure 4

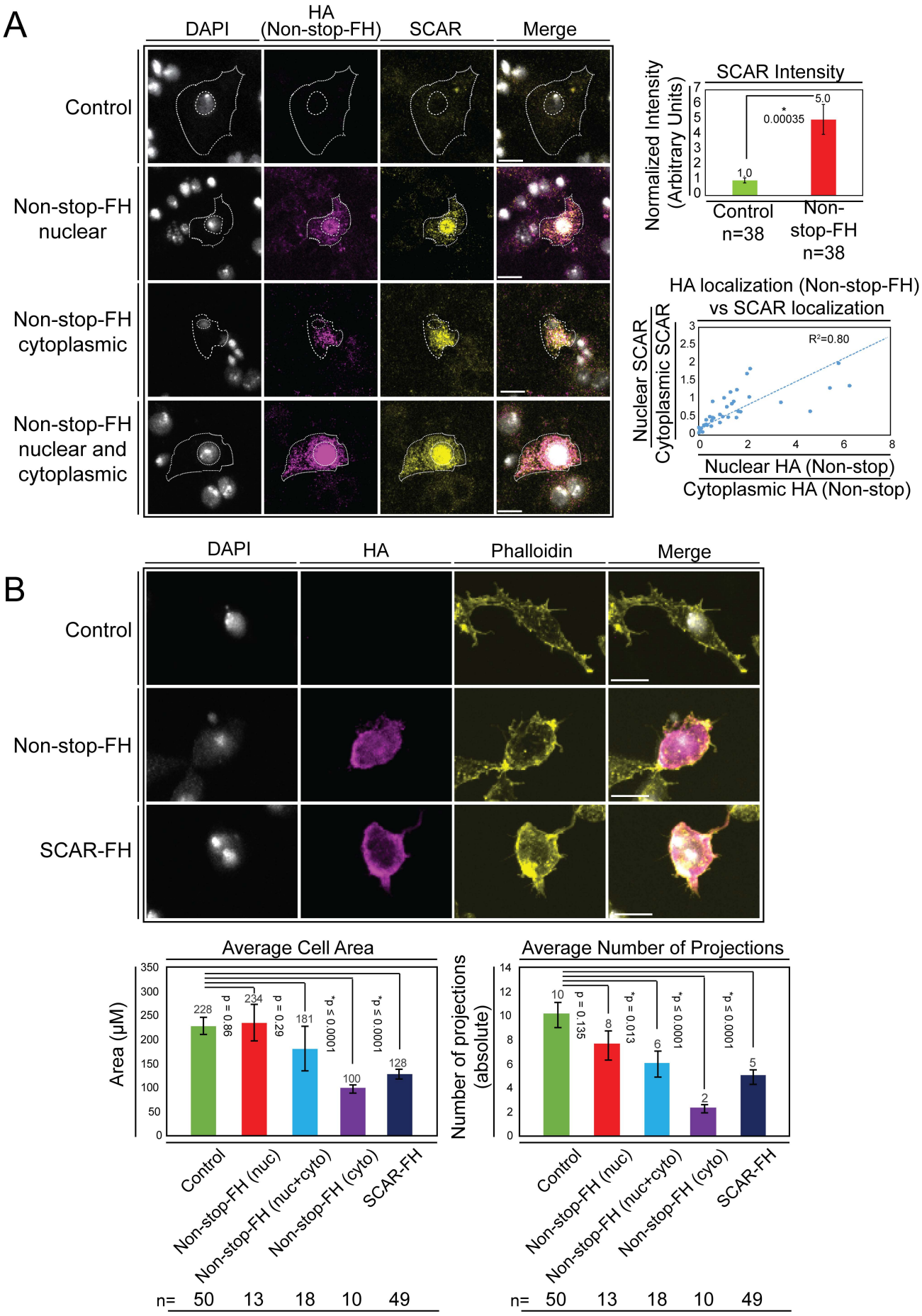

A
DAPI
HA (Non-stop-FH)
SCAR
Merge
Control
Non-stop-FH nuclear
Non-stop-FH cytoplasmic
Non-stop-FH nuclear and cytoplasmic
SCAR Intensity
Normalized Intensity (Arbitrary Units)
5.0
0.00035
1.0
Control n=38
Non-stop-FH n=38
HA localization (Non-stop-FH) vs SCAR localization
Nuclear SCAR / Cytoplasmic SCAR
R²=0.80
Nuclear HA (Non-stop) / Cytoplasmic HA (Non-stop)
B
DAPI
HA
Phalloidin
Merge
Control
Non-stop-FH
SCAR-FH
Average Cell Area
Area (μM)
228
234
181
100
128
p = 0.86
p = 0.29
p ≤ 0.0001
p ≤ 0.0001
Control
Non-stop-FH (nuc)
Non-stop-FH (nuc+cyto)
Non-stop-FH (cyto)
SCAR-FH
n= 50 13 18 10 49
Average Number of Projections
Number of projections (absolute)
10
8
6
2
5
p = 0.135
p = 0.013
p ≤ 0.0001
p ≤ 0.0001
Control
Non-stop-FH (nuc)
Non-stop-FH (nuc+cyto)
Non-stop-FH (cyto)
SCAR-FH
n= 50 13 18 10 49

Figure 5

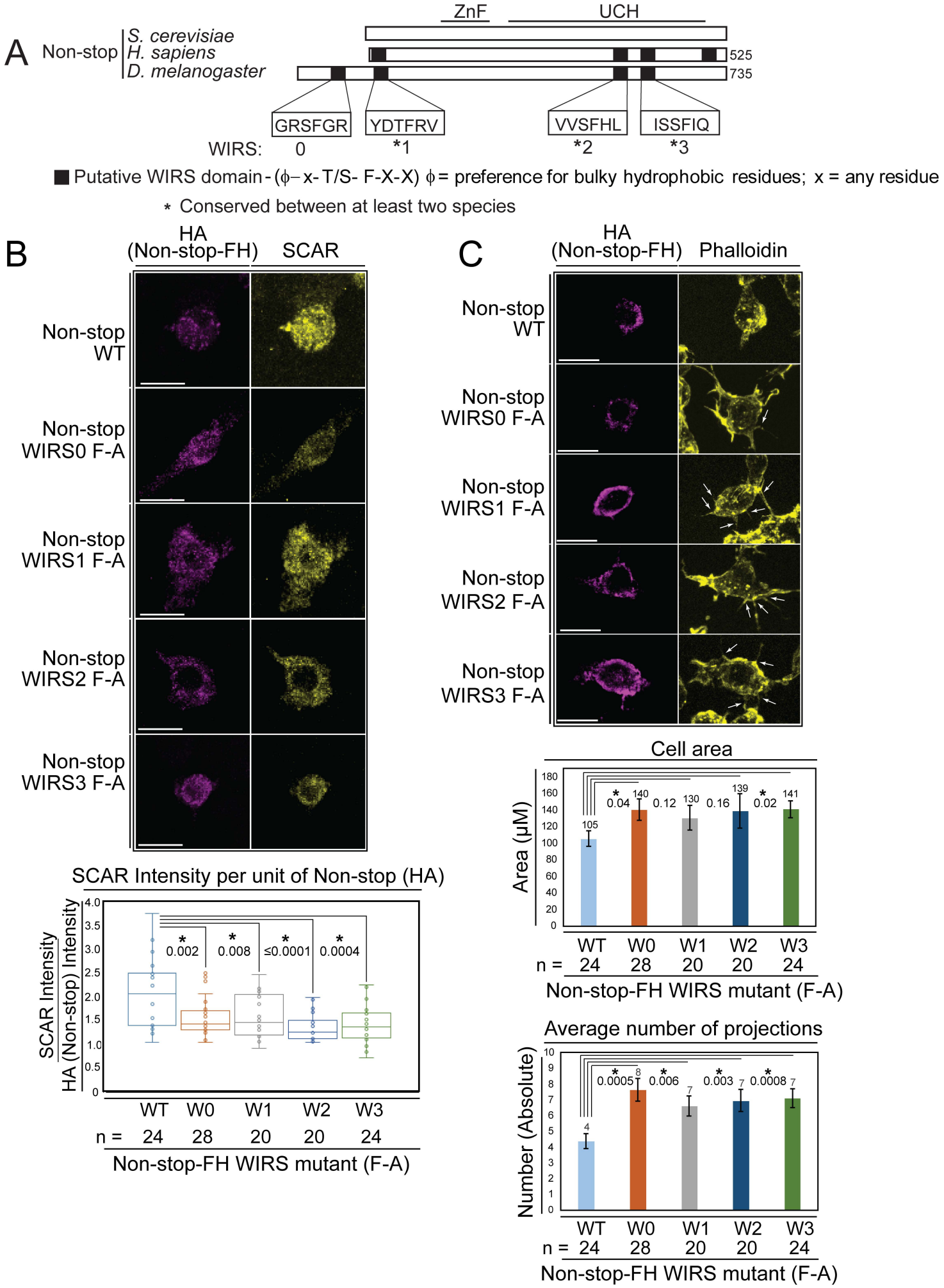
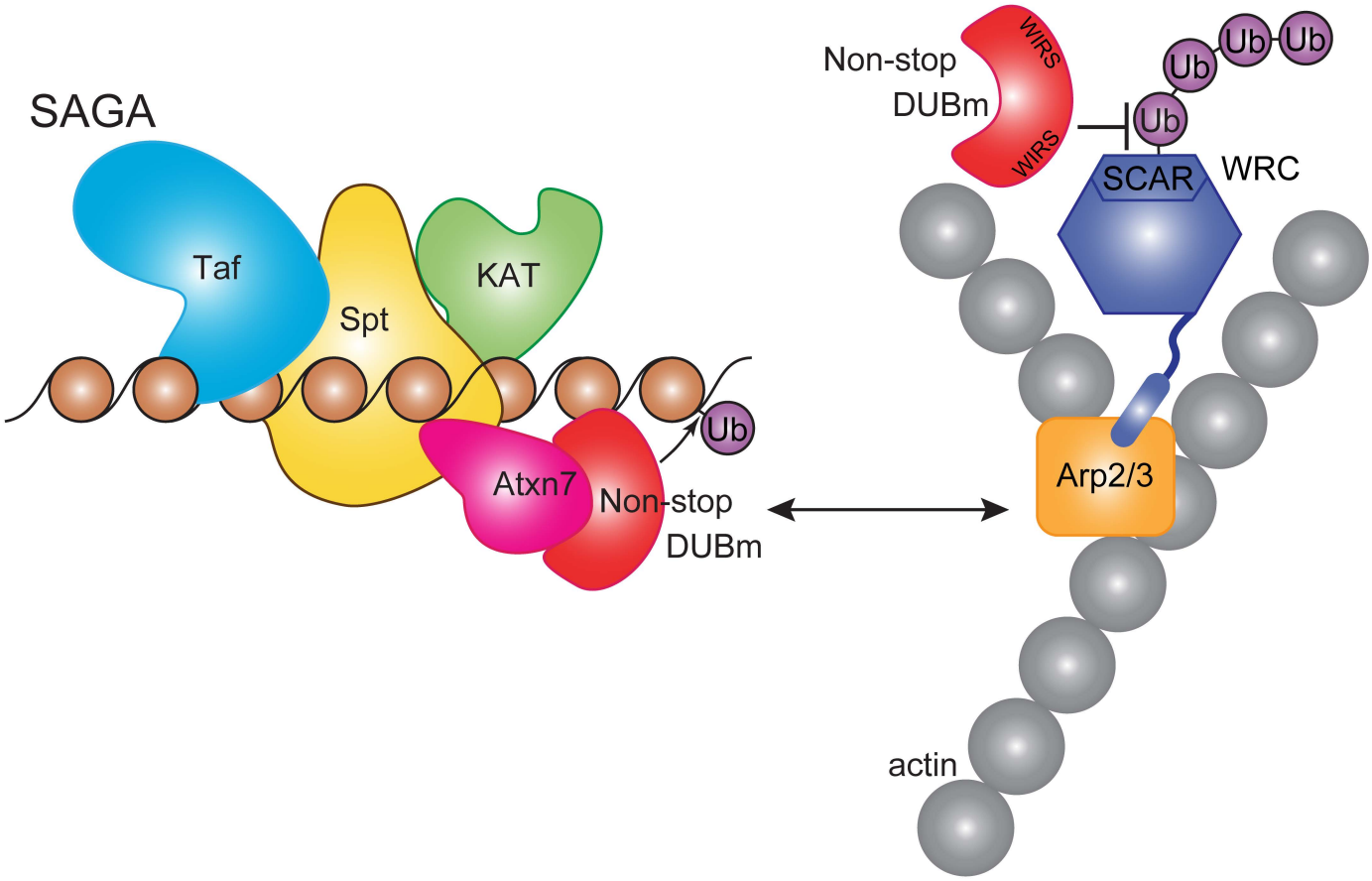

A
Non-stop
S. cerevisiae
H. sapiens
D. melanogaster
ZnF
UCH
525
735
GRSFGR
YDTFRV
VVSFHL
ISSFIQ
WIRS:
0
*1
*2
*3
Putative WIRS domain - (ϕ−x-T/S- F-X-X) ϕ = preference for bulky hydrophobic residues; x = any residue
* Conserved between at least two species
B
HA (Non-stop-FH)
SCAR
Non-stop WT
Non-stop WIRS0 F-A
Non-stop WIRS1 F-A
Non-stop WIRS2 F-A
Non-stop WIRS3 F-A
SCAR Intensity per unit of Non-stop (HA)
SCAR Intensity / HA (Non-stop) Intensity
* 0.002
* 0.008
* ≤0.0001
* 0.0004
WT W0 W1 W2 W3
n = 24 28 20 20 24
Non-stop-FH WIRS mutant (F-A)
C
HA (Non-stop-FH)
Phalloidin
Non-stop WT
Non-stop WIRS0 F-A
Non-stop WIRS1 F-A
Non-stop WIRS2 F-A
Non-stop WIRS3 F-A
Cell area
Area (μM)
105
* 0.04
140
0.12
130
0.16
139
* 0.02
141
WT W0 W1 W2 W3
n = 24 28 20 20 24
Non-stop-FH WIRS mutant (F-A)
Average number of projections
Number (Absolute)
4
* 0.0005
8
* 0.006
7
* 0.003
7
* 0.0008
7
WT W0 W1 W2 W3
n = 24 28 20 20 24
Non-stop-FH WIRS mutant (F-A)

Figure 6


SAGA
Taf
Spt
KAT
Atxn7
Non-stop
DUBm
Ub
Non-stop
DUBm
WIRS
WIRS
Ub
Ub
Ub
Ub
SCAR
WRC
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