***Drosophila* p38 MAPK Interacts with BAG-3/starvin to Regulate Age-dependent**

Protein Homeostasis

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Summary

As organisms age, they often accumulate protein aggregates that are thought to be

toxic, potentially leading to age-related diseases. This accumulation of protein

aggregates is partially attributed to a failure to maintain protein homeostasis. A variety of

genetic factors have been linked to longevity, but how these factors also contribute to

protein homeostasis is not completely understood. In order to understand the

relationship between aging and protein aggregation, we tested how a gene that

regulates lifespan and age-dependent locomotor behaviors, p38 MAPK (p38Kb),

influences protein homeostasis as an organism ages. We find that p38Kb regulates age-

dependent protein aggregation through an interaction with the Chaperone-Assisted

Selective Autophagy complex. Furthermore, we have identified Lamin as an age-

dependent target of p38Kb and the Chaperone-Assisted Selective Autophagy complex.

Introduction

Protein turnover is critical for maintaining tissue health as many proteins become

damaged or misfolded during normal tissue functions. Therefore, the cell utilizes a

variety of protein quality control mechanisms to refold or degrade these damaged

proteins, including the ubiquitin proteasome system and macroautophagy. During aging,

protein quality control mechanisms become less efficient leading to the accumulation of

damaged or misfolded proteins that begin to form protein aggregates[1]. It has been

hypothesized that these aggregates are toxic and may lead to the deleterious

phenotypes associated with normal aging, such as impaired tissue function[1].

Furthermore, decreased protein aggregation has been associated with longevity. For

example, over-expression of Foxo leads to an increased lifespan but also a concordant

decrease in protein aggregation in *C. elegans*, *Drosophila,* and mice [2–6], suggesting that

lifespan and protein aggregation are tightly linked processes. However, the molecular

mechanisms that underlie the relationship between aging and protein homeostasis have

not been fully characterized.

One pathway that has been linked to both aging and protein homeostasis is the

stress response p38 MAPK (p38K) pathway. In mammals, there are four p38K genes (α,

β, γ, and δ), and p38Kα has been linked to both the inhibition [7,8] and induction [9,10] of

macroautophagy, in particular in response to oxidative stress [11,12]. In addition, p38Kα

has been linked to regulating macroautophagy in cellular senescence [13–15]. However,

how p38K signaling may contribute to protein homeostasis in response to natural aging

is not well understood. The fruit fly *Drosophila melanogaster* has two canonical p38K

genes (p38Ka and p38Kb), and we have previously reported that p38Kb acts in the adult

musculature to regulate aging. We found that over-expression of p38Kb leads to

increased lifespan while loss of p38Kb results in a short lifespan and age-dependent

locomotor behavior defects [16]. In addition, oxidatively damaged proteins accumulate in

the muscle of p38Kb mutants with age [16], and loss of p38Kb leads to increased

polyubiquitination of insoluble proteins and alterations in oxidative stress dependent

translation [17], suggesting that these oxidatively damaged proteins may be aggregating in

p38Kb mutants. Furthermore, p38Kb has been shown in a *Drosophila* cell culture system

to pull down with the HspB8 homologue CG14207 [18], which plays a role in the muscle by

regulating protein homeostasis as a part of a protein quality control mechanism called

BAG-3 Mediated Selective Macroautophagy pathway in mammalian systems or the

Chaperone-Assisted Selective Autophagy (CASA) complex in flies [19,20]. The CASA

complex also includes the chaperone Hsc70 and the co-chaperone BAG-3 (starvin in

flies). BAG-3/starvin binds to specific damaged or misfolded protein substrates and

brings them to the CASA complex where it binds to Hsc70 and HspB8. Those substrates

that cannot be refolded by the complex are polyubiquitinated and targeted to the

autophagosome through a handover between BAG-3/stv and the autophagy adaptor

protein p62 (ref(2)p in flies), and subsequently degraded through the autophagosome-

lysosome system [21–25].

Here, we report that p38Kb regulates age-dependent muscle protein

homeostasis through an interaction with the CASA complex. We find that p38Kb acts as

an intermediary between BAG-3/starvin and p62/ref(2)p in targeting damaged or

misfolded proteins for degradation. This interaction is not only important for maintaining

protein homeostasis but also for lifespan extension. In addition, we find that the

Drosophila homologue of the Hutchinson-Gilford progeria protein, Lamin A/C, is a target

for p38Kb and CASA complex mediated protein turnover, suggesting that the p38Kb

aging phenotypes may be a result of impaired Lamin degradation.

Materials and Methods

Genotypes

UAS-p38Kb wt, UAS-p38Kb Kinase Dead, p38Kb$^{\Delta45}$, p38Kb^{Ex41}, w^{1118}, Mef2-GAL4 and

MHC-GAL4 were as described in [16]. The p38Kb^{Ex41} is a precise excision allele and

serves as a genetic background control for p38Kb$^{\Delta45}$ deletion mutation.

UAS-stv RNAi 34408 (w[1118]; P{GD10796}v34408) and UAS-stv RNAi 34409 (w[1118];

P{GD10796}v34409/TM3) are described in [26] and are from the Vienna Drosophila

Resource Center.

stv-GFP trap (w[11118];; Pbac{754.P.F3v30} stv$^{+CPTI\ 002824}$), HspB8-GFP trap (w[1118]

PBac{810.P.FSVS-2}CG14207^{CPTI004445}), Hsc70-4 GFP trap (*w[1118] PBac{544.SVS-*

1}N^{CPTI002347}) are from the Kyoto Stock Center.

w[1118]; P{y[+mDint2] w[BR.E.BR]=SUPor-P}ref(2)P^{KG00926}, w[1118];;+mDint2 EY4969 stvEP,

Lam^{A25} pr[1], P{UAS-mito-HA-GFP.AP}3, w[126]; P{w[+mC]=UAS-Hsc70-4.WT}B, and

w[1118]; P{w[+mC]=UAS-Lam.GFP}3-3 were obtained from the Bloomington Drosophila

Stock Center.

All fly stocks were backcrossed into the w[1118] background and isogenized for 10

generations. All stocks were reared at 25ºC in a 12hr:12hr light:dark cycle on standard

fly food media.

Immunofluorescence

Adult flies were fixed in 4% paraformaldehyde for 48hrs at 4°C. Indirect flight muscles

were dissected in 1X PBS, permeabilized in 1X PBS 0.15% Triton-X 100, and blocked in

NGS + 0.15% Triton-X 100. Samples were incubated in primary antibody at 4°C

overnight, washed in 1X PBS 0.15% Triton-X 100, and incubated in secondary antibody

at room temperature for 2hrs. Samples were mounted in Vectashield mounting medium

(Vectorlabs) and visualized using a laser scanning confocal microscope. Antibodies:

rabbit anti-GFP 1:400 (Invitrogen), mouse anti-FLAG M2 1:1000 (Sigma), rabbit anti-stv

1:1000 (gift of Jög Höhfeld), rat anti-α actinin 1:100 (Abcam), rabbit anti-ubiquitin

linkage-specific K63 anti-mouse 1:200 (Abcam), IgG- Alexa Fluor 488 1:200 (Life

Technologies), anti-mouse IgG- Alexa Fluor 568 1:500 (Life Technologies), anti-rabbit

IgG- Alexa Fluor 488 1:500 (Life Technologies) and Rhodamine Phalloidin 1:2000.

Protein aggregate analysis

Indirect flight muscles were prepared as described above from 9 individual flies per

genotype. Protein aggregates were identified using mouse anti-polyubiquitin 1:1000

(Enzo Life Sciences). Three muscles from each individual fly were imaged as z-series

and flattened into a single image as a max projection using confocal microscopy for a

total of 27 muscles per genotype. Images were analyzed using Image J "Analyze

Particles" function with a diameter of 100 pixels set for the minimum aggregate size.

Aggregate number and size were analyzed using ANOVA followed by Tukey's HSD

using the R package "multcomp" to generate significance groups with each letter group

being significantly different with a p value of ≤ 0.05. Within genotype/across time point

analyses were performed using the Welch two sample t-test in R.

Lifespan

For lifespan experiments using the UAS-p38Kb^{wt}, ref(2)p -/+, p38Kb$^{\Delta 45/\Delta 45}$, the stv EP

(stv^{wt}) lines, and their respective controls, virgin females were kept on standard

molasses *Drosophila* media. Due to a change in lab food, the stv RNAi 34408 and stv

RNAi 34409 lifespan experiments (with their respective controls) were performed on the

standard Bloomington *Drosophila* media. Virgin flies were collected and reared at 25°C

in a 12hour:12hour light:dark cycle. Flies were put on new food twice a week. The

number of dead animals was scored daily. Lifespan was analyzed using a log rank test

to compare genotypes with censored data on all genotypes and then on all pairwise

comparisons using the R package "survival" with Benjamini and Hochberg correction

(false discovery rate < 0.05).

Co-immunoprecipitation

Flies were aged 1 week or 5 weeks. Forty thoraxes per genotype per condition were

homogenized in high salt buffer (0.5 M KCl, 35% glycerol,10 mM HEPES pH 7.0, 5 mM

$MgCl_2$, 0.5 mM EDTA pH 8.0, 0.1% NP40 25 mM NaF, 1 mM Na_2VO_4, 1 mM DTT,

Complete protease inhibitor). The lysate was flash frozen in liquid nitrogen and quickly

thawed at 37°C. Then lysates were rocked at 4°C for 30 minutes and centrifuged at

14,200 x g for 30 minutes at 4°C. The supernatant was transferred to equilibrated beads

anti-Flag (M2) agarose (Sigma) or anti-GFP agarose (Chromotek) and rocked for 2

hours at 4°C. Beads were collected using a magnetic bar and washed four times with IP

buffer (50 mM HEPES pH 7.0, 100 mM KCl, 0.4% NP40 1.5 mM $MgCl_2$, 5% glycerol, 25

mM Na, 1 mM Na_2VO_4, 1 mM EDTA, 1 mM DTT, Complete protease inhibitor). Lysates

were then analyzed by immunoblotting using rabbit anti- GFP 1:1000 (Invitrogen),

mouse anti-FLAG M2 1:1000 (Sigma), rabbit anti-phospho-p38 1:1000 (Cell Signaling

Technologies), goat anti total p38 1:1000 (Santa Cruz Biotechnology), rabbit anti-stv

1:10,000 (gift of Jrög Höhfeld) or mouse anti-Lamin 1:1000 (DHSB).

Immunoblotting

Wild type flies (w^{1118}) were aged either for 3, 15, 30, and 45 days or for 1-5 weeks. Three

thoraxes were dissected and homogenized in 1x Laemmli buffer. Immunoblots were

performed as described in [16]. Membranes were developed using SuperSignal West

Femto kit (ThermoFisher) or Pierce ECL (ThermoFisher) and exposed on

autoradiography film. Antibodies used were: rabbit anti-GFP 1:1000 (Invitrogen), rabbit

anti-starvin 1:10,000 (gift of Jrög Höhfeld), mouse anti-actin 1:5,000,000 (Sigma), mouse

anti-FLAG M2 (Sigma), rabbit anti-alpha tubulin (Cell Signaling Technologies), mouse

anti-Lamin 1:100 (DHSB), rabbit anti-phospho Lamin A Ser22 1:1000 (Thermofisher),

mouse anti-beta tubulin (E-10) 1:5000 (Santa Cruz Biotechnology), mouse anti- HRP

1:20,000 (Jackson Labs), rabbit anti-HRP 1:40,000 (Jackson Labs). Densitometry was

performed using a minimum of three independent blots. For statistical analysis of protein

expression level, pixel density of the tested protein was normalized within sample to the

loading control. These values were then normalized to control to calculate fold change.

The fold change values were analyzed by Student's t-test or ANOVA [27] as appropriate.

Sucrose Gradient Fractionation

p38Kb$^{Ex41/Ex41}$ and p38Kb$^{\Delta45/\Delta45}$ flies were aged three weeks. 30 thoraxes per genotype

were dissected and homogenized in NP40 lysis buffer. Samples were centrifuged at

800xg for 10 minutes at 4°C. The supernatant was then transferred to a 15-50% sucrose

discontinuous gradient. Samples were then ultracentrifuged at 55,000 rpm (201,000xg at

r_{av}) for 20 hours at 4°C in a TLS-55 in a Beckman Coulter Optima TLX Ultracentrifuge.

200µl fractions were collected and the pellet was resuspended in an equal volume of

NP40 Lysis Buffer.

Stv localization

Immunohistochemistry on p38Kb$^{Ex41/ Ex41}$ and p38Kb$^{\Delta45/\Delta45}$ Indirect flight muscles was

performed as described above. Confocal images from five individual flies per genoytpe

were analyzed for average pixel density using ImageJ in three different non-overlapping

locations on each muscle for a total of 15 measurements per genotype. Average pixel

density was analyzed by Student's t-test using R.

Results

p38Kb regulates age-dependent protein homeostasis.

p38Kb null mutant animals (p38Kb$^{\Delta45/\Delta45}$, a deletion of the p38Kb gene) exhibit age-

dependent locomotor behavior defects and have a 48% lifespan reduction. In addition,

biochemical analysis suggests that p38Kb null mutants have increased levels of

insoluble polyubiquitinated proteins in the thoracic musculature of aged animals as

observed by immunoblot analysis [17]. However, protein aggregate size and distribution

have not been visualized in the p38Kb mutants, nor is it known whether augmenting

p38Kb activity in muscles leads to a change in protein homeostasis. Therefore, we

analyzed how p38Kb expression influences protein aggregation. We find that p38Kb null

mutants have an increased number of protein aggregates in the adult indirect flight

muscle at 1 week and 3 weeks of age (Figure 1A-B and E-F, Table S1) and increased

aggregate size with age (Figure 1G-H, Table S1) as compared to a genetic background

control (p38Kb$^{Ex41/Ex41}$, a precise excision allele). Furthermore, transgenic inhibition of

p38Kb in the muscle using a dominant negative kinase dead construct (p38Kb^{KD}, [16]) also

results in a significant increase in aggregate number, however aggregate size was not

affected (Figure 1I-L and Table S2). Conversely, as both strong and moderate levels of

p38Kb over-expression lead to an increased lifespan (37% and 14% extension,

respectively) [16], we tested if p38Kb over-expression also affects protein aggregation. We

find that both strong over-expression of p38Kb in the adult muscle using the Mef2-GAL4

driver (Figure 1C-D and M-P, Table S3) and moderate over-expression of p38Kb using

the MHC-GAL4 driver (Figure 1Q-T and Table S4) leads to decreased protein aggregate

number and size throughout the lifespan. It has been hypothesized that protein

aggregate accumulation and increased size may be toxic, potentially explaining the

decreased lifespan and age-dependent locomotor abnormalities in p38Kb$^{\Delta45}$ null mutants

and the increased longevity in the p38Kb over-expression animals.

p38Kb mediates age-dependent phenotypes through autophagy

In order to determine what mechanism plays a role in the clearance of polyubiquitinated

protein aggregates, we first tested what type of ubiquitin linkage is present in the

aggregates in wild type flies. In particular, K63-linked ubiquitination has been shown to

facilitate the formation of aggregates [28–30] that are cleared through autophagy [30–32]. We

find that a subset of aggregates from both aged wild type and p38Kb mutant flies contain

K63-linked ubiquitinated proteins (Figure 2), suggesting that these muscle protein

aggregates are degraded through autophagy.

Polyubiquitinated protein aggregates can be degraded through selective

autophagy in which the adaptor protein p62 (ref(2)p in flies) promotes the packaging and

delivery of polyubiquitinated proteins to the autophagosome[33]. If p38Kb requires

selective autophagy to mediate protein homeostasis, then loss of ref(2)p will block the

p38Kb mediated decreased aggregation phenotype. We find that loss of a single copy of

ref(2)p results in fewer aggregates (Figure 2G-H and Table S5), which may reflect

compensation by other protein clearance mechanisms with aging, especially as

homozygous *ref(2)p* mutants are viable. When a single copy of *ref(2)p* is removed in the

p38Kb over-expression background, this prevents the reduced protein aggregation

observed in the p38Kb over-expression animals at both young and old ages (Figure 2G-

H and Table S5). This dominant interaction suggests that *ref(2)p* acts downstream of

p38Kb to promote the degradation of protein aggregates.

p38Kb colocalizes with the CASA complex in the adult flight muscle.

In order to better understand the role of p38Kb in protein homeostasis, we first

determined where in the muscle p38Kb localizes and find that a FLAG-tagged p38Kb

(green) colocalizes with the Z-disc marker alpha-actinin and is also present at the M-line

(Figure 3A). The muscle Z-disc is an area of high protein turnover, and one protein

quality control mechanism that localizes to the Z-disc in mice and adult flies is the

Chaperone-Assisted Selective Autophagy (CASA) complex (known as the BAG-3

mediated selective autophagy complex in mammals)[19,34,35]. In adult *Drosophila* muscle,

the CASA complex has been reported to consist of the Hsc70 homologue Hsc70-4, the

HspB8 homologue CG14207 and the BAG-3 homologue starvin (stv) [19]. We find that

p38Kb colocalizes with all the CASA complex members at the Z-disc and M-line (Figure

3B-D).

p38Kb physically interacts with the CASA complex in the adult flight muscle.

In order to further determine if p38Kb physically interacts with the CASA complex in the

adult muscle, we performed co-immunoprecipitation experiments. In *Drosophila* cell

culture p38Kb[wt] was shown to co-immunoprecipitate with the HspB8 homologue

CG14207[18], therefore, we tested if this interaction also occurs in adult indirect flight

muscle. As the interaction between p38Kb and its targets may be transient, we utilized a

FLAG-tagged p38Kb kinase dead construct (UAS-p38Kb[KD] Mef2-GAL4) that is able to

be activated and bind to a target but cannot phosphorylate it, leading to a delayed

release of the target[36]. We expressed p38Kb[KD] in the adult flight muscle of a fly that also

endogenously expressed GFP tagged HspB8, in which GFP is spliced in as a new exon

of the endogenous gene. This results in a GFP fusion protein that is expressed in the

same pattern as endogenous HspB8 ([19] and Figure 3C'). We find that p38Kb[KD] was able

to pull down HspB8 (Figure 3E) as compared to the HspB8-GFP control background. In

addition, expression of p38Kb[KD] in a wildtype background was able to pull down un-

tagged endogenous stv (Figure 3F).   We then performed the reverse

immunoprecipitation experiments in which we immunoprecipitated control GFP or

endogenously GFP tagged Hsc70-4, HspB8 or stv from muscle lysates and probed for

endogenous phosphorylated p38K, which is the active form of p38K. We find that all

three members of the CASA can co-immunoprecipitate with endogenous phosphorylated

p38K in the muscle (Figure 3G, Figure S1D). We repeated these experiments in which

we expressed the FLAG tagged p38Kb^{KD} and found that p38Kb co-immunoprecipitates

with the CASA complex (Figure S1A-C) with stronger binding at younger ages than older

ages. To determine if this reduction in binding was due to an age-dependent decrease in

the ability of p38K to associate with the CASA complex, we tested if the expression

levels of either the CASA complex or the FLAG tagged p38Kb^{KD} are changed with age.

We found no significant difference in the expression levels of Hsc70-GFP and stv-GFP

with age (Figure S2A and C) while levels of HspB8-GFP increased with age (Figure

S2B). In addition, the levels of over-expressed p38Kb^{KD} declined with age (Figure S2D).

We also have found that levels of p38K decline with age[37]. These data suggest that the

levels of p38Kb protein may be limiting for CASA complex functions as the fly ages.

p38Kb acts downstream of the CASA complex.

We next tested the relationship between p38Kb and the CASA complex to

determine if p38Kb acts either upstream or downstream of the CASA complex to

promote protein homeostasis. As BAG-3/stv provides both the specificity to the CASA

complex and is involved in the handoff of damaged proteins to p62/ref(2)p for autosomal

degradation [21–25], we tested for genetic interactions between p38Kb and *stv*. *stv* null

mutants have impaired locomotor functions, muscle degeneration and early lethality [19,38].

Due to the severity of these null phenotypes, we utilized *stv* RNAi lines to generate an

allelic series of *stv* loss of function in the muscle. We find that weak inhibition of stv

(UAS-stv RNAi[34408] MHC-GAL4) had no effect on protein aggregation (Table S6).

However, moderate inhibition of stv (UAS-stv RNAi[34409] MHC-GAL4) results in increased

protein aggregate number and size (Figure 4A-B and Table S7) and results in a

decrease in lifespan particularly in the first half of life as compared to outcrossed

controls (Figure 4C and Table S8). Strong inhibition of *stv* (UAS-stv RNAi[34408] Mef2-

GAL4) leads to a severely reduced lifespan of ~4 days on average (Figure 4D and Table

S9).

If p38Kb acts to regulate the downstream activity of the CASA complex, then

over-expression of p38Kb should rescue these *stv* RNAi phenotypes. We find that over-

expression of p38Kb rescues both protein aggregate number and size in the *stv*

moderate inhibition background (Figure 4A-B and Table S7) and also rescues the

lifespan defect (Figure 4C and Table S8). Furthermore, p38Kb over-expression is able to

partially rescue the decreased lifespan exhibited by strong *stv* inhibition increasing

average lifespan from 4 days to 13 days (Figure 4D and Table S9). To further test if

p38Kb acts downstream of the CASA complex, we combined loss of p38Kb with CASA

complex over-expression. If p38Kb acts downstream of the CASA complex, then over-

expression of the CASA complex members in the p38Kb mutant background will not be

able to rescue the p38Kb mutant phenotypes. We find that over-expression of Hsc70-4

in the muscle fails to rescue the p38Kb mutant shortened lifespan (Figure S3A, Table

S10), even though this extends lifespan in a wild type background (Figure S3B, Table

S11). Furthermore, we find that over-expression of stv in the p38Kb mutants not only

fails to rescue the p38Kb mediated short lifespan defect but reduces the lifespan further

(Figure 4E and Table S12). This is particularly striking as over-expression of stv in a wild

type background does not significantly affect lifespan (Figure 5E and Table S13) as

compared to the outcrossed transgene control. These data in combination with the

decrease of p38K binding to the CASA complex with age when p38K levels are reduced

suggest that p38Kb may be a limiting factor in regulating the downstream activity of the

CASA complex in which it hands the poly-ubiquitinated misfolded proteins to ref(2)p for

degradation through the autophagosome.

p38Kb regulates the activity of the CASA complex in protein homeostasis.

If p38Kb is a limiting factor for stv function, then the combined over-expression of

p38Kb and stv may result in a further beneficial effect. We find that over-expression of

stv alone results in fewer aggregates at young and old ages (Figure 5A-B and Table

S14) and smaller aggregates with age (Figure 5D and Table S14) as compared to

outcrossed controls. However, co-over-expression of p38Kb and stv does not result in a

further reduction in aggregate number as compared to over-expression of p38Kb or stv

alone (Figure 5A and Table S14). By 5 weeks of age, co-over-expression of p38Kb and

stv flies have comparable aggregate number to controls (Figure 5B and Table S14).

Conversely, p38Kb and stv co-over-expression results in a reduction in aggregate size at

a young age compared to over-expression of stv alone (Figure 5C and Table S14),

suggesting that p38Kb is a limiting factor for stv function in regulating aggregate size.

Unlike with aggregate number, the aggregates remain significantly smaller in size as the

flies age in the combined over-expression background (Figure 5D and Table S14). We

also find that co-over-expression of p38Kb and stv leads to an additional 5% increase in

lifespan relative to p38Kb over-expression alone (Figure 5E and Table S13).

Interestingly, the co-over-expression animals show a very similar lifespan to the p38Kb

over-expression alone animals until ~day 50, when the p38Kb alone animals begin to die

at a faster rate (Figure 5E). These data suggest that co-over-expression of p38Kb and

stv provides beneficial effects in early adulthood that continue throughout adulthood

leading to increased lifespan despite the presence of protein aggregates. Another

possibility is that aggregate size and/or content may play a more important role in

determining lifespan as compared to overall aggregate number.

p38Kb is required for proper localization of stv in the muscle.

Since we find that p38Kb acts between stv and ref(2)p in regulating protein

aggregation, we hypothesize that p38Kb plays a role in stabilizing the transfer of

misfolded proteins primed for degradation from the CASA complex to ref(2)p. In support

of this hypothesis, we find that over-expression of p38Kb leads to increased efficiency in

targeting misfolded proteins to the autophagosome (Figure 1M-T), while inhibition of

p38Kb results in increased protein aggregation (Figure 1E-L). In addition, loss of p38Kb

may lead to a failure of stv to maintain its interaction with the CASA complex or to

interact with ref(2)p. Therefore, we tested if stv is still able to localize to the Z-disc in the

absence of p38Kb. We find that in p38Kb null mutants, stv protein levels are increased

(Figure 5F), and stv is more diffusely located throughout the muscle but is still able to

localize to the Z-disc and M-line in (Figure 5G-I), suggesting that its localization is

partially impaired in the absence of p38Kb. Furthermore, we find that the localization of

the CASA complex member HspB8 in the muscle is unaffected by loss of p38Kb (Figure

S3C-D). These data indicate that p38Kb may play a role in stabilizing the interaction

between the CASA complex and stv that allows stv to direct misfolded proteins to

ref(2)p.

Lamin protein binds to the CASA complex and accumulates in stv RNAi and

p38Kb mutants.

In order to determine if p38Kb might be playing a role in the targeting of

misfolded proteins from stv to ref(2)p, we first needed to identify a protein target of both

stv and p38Kb. Therefore, we focused on proteins associated with Limb-Girdle Muscular

Dystrophy (LGMD), which is caused by mutations in either BAG-3 (stv, myofibrillar

myopathy-6) or HspB8 (LGMD Type 1D) [39-41]. In addition, p38K signaling has been

implicated in LGMD [42-44]. *Drosophila* have 19 orthologues of LGMD proteins, one of

which is Lamin A/C (Lamin or Lamin Dm0 in flies). Lamin is of particular interest since

mutations in Lamin A/C also result in the accelerated aging disorder Hutchinson-Gilford

progeria [45-47], and Lamin has been shown to aggregate under oxidative stress conditions

[48]. Mutations in Lamin A/C are also sufficient to induce Lamin aggregation and abnormal

nuclear morphology in human cell culture, *C. elegans*, and *Drosophila* systems [46,49-57]. In

addition, Lamin proteins are highly post-translationally modified, resulting in changes in

its solubility[56,58-63]. Furthermore, in *Drosophila* inhibition of either fly homologue of Lamin

A/C have similar phenotypes to p38Kb and/or stv mutants, such as reduced locomotor

function and increased activity of the Nrf-2/Keap-1 pathway [51,64,6557,66]. In addition, it has

recently been shown that BAG-3, one of the mammalian homologues of stv, can target

nuclear Lamin B, a paralogue of Lamin A/C, for degradation [67].

     If Lamin is a target of the CASA complex, then decreased activity of the CASA

complex should result in an accumulation of Lamin protein. Flies have two homologues

of Lamin A/C, Lamin Dm_0 and LamC. We find that the levels of Lamin Dm_0, do not

change with age (Figure 6A), however, inhibition of stv results in a significant increase in

the total amount of Lamin Dm_0 protein regardless of age (Figure 6A-B). Interestingly,

loss of p38Kb leads to an age-dependent increase in Lamin Dm_0 protein as compared to

age matched controls (Figure 6C-D). These data in addition to our findings that p38Kb is

limiting for stv localization (Figure 5H) and that the levels of p38Kb decline with age[37]

suggest that p38Kb activity later in life is important for maintaining protein homeostasis

and lifespan.

     To investigate if Lamin Dm_0 is accumulating in the protein aggregates, we

performed fractionation experiments in which we find that in controls Lamin Dm_0 is

predominantly found in fractions 5-7 and also in the aggregate containing pellet (Figure

7A), which is characterized by an increased amount of poly-ubiquitinate proteins (Figure

7C). Interestingly, we also find a low molecular weight from of Lamin Dm_0 mainly

restricted to fraction 5 and a higher molecular weight form that is predominantly in the

pellet (Figure 7A). We next tested how loss of p38Kb affects the aggregation of Lamin

Dm_0 and find that p38Kb mutants have decreased Lamin Dm_0 in fractions 5-7 with a

concurrent increase of both the main and high molecular weight species of Lamin Dm_0 in

the pellet (Figure 7A). This suggests that Lamin Dm_0 is a target of p38Kb and that loss of

p38Kb results in increased aggregation of Lamin Dm_0.

Of interest is this high molecular weight form of Lamin Dm_0, which is present in

the aggregates (Figure 7A). Lamin proteins are highly post-translationally modified[56,58–

63], and one important protein domain in Lamins is the CaaX box, which is farnesylated

and required for Lamin localization to the inner nuclear membrane[63,68–70]. To test if this

might be a farnesylated form of Lamin Dm_0, we utilized the Lam^{A25} mutant which has a

frameshift that results in the loss of the C-terminal CaaX box[71]. We find that the high

molecular weight form of Lamin Dm_0 is lost in the Lam^{A25} mutant whereas the

predominant 75kDa band of Lamin Dm_0 is still present (Figure 7D). These data suggest

that the high molecular weight Lamin Dm_0 we observe is the farnesylated form. We do

not observe a consistent effect on levels due to either stv inhibition or loss of p38Kb,

which suggest that p38Kb mediates farnesylated Lamin Dm_0 aggregation than overall

levels.

In addition to farnesylation, Lamins can also be phosphorylated by a variety of

kinases that can change the solubility of Lamin porteins[56,72]. For example, in a

Drosophila cell culture system, a phospho-mimic mutation at Ser45 (the equivalent of

Ser22 in humans) results in Lamin aggregation in both the nucleus and cytoplasm [56].

This serine residue is phosphorylated by the cd2 kinase [73], but is also a predicted p38K

phosphorylation site. As the antibody epitope is conserved in both Lam Dm_0 and LamC,

we may not be able to detect specific changes in phosphorylated Lam Dm_0 versus

LamC, however, we can assess changes in the levels of Lamin phosphorylation in

general. We find that phospho-Lamin levels are not significantly altered in the p38Kb

mutants (Figure 6E-F), suggesting that p38Kb is not required for Ser45 phosphorylation.

Furthermore, this phosphorylated form of Lamin runs at lower molecular weight than the

main form of Lamin (Figure 7D top and middle panels), suggesting that Lamins in adult

Drosophila muscle is further processed. As this phosphorylation site is near the epitope

recognized by the total lamin antibody, it maybe that lamin phosphorylation obscures the

ability of this antibody to detect this form of Lamin.

We next examined if phosphorylated Lamin is also present in the aggregates. We

find that in controls, phosphorylated Lamin is present in factions 5-7 with low amounts in

the pellet (Figure 7B). We also observe a low molecular weight phospho-Lamin form that

appears in the pellet (Figure 7B). In the p38Kb mutants, phospho-Lamin expression is

reduced in fractions 6 and 7 and accumulates in the pellet (Figure 7B). Furthermore,

increasingly smaller low molecular weight species of phospho-Lamin are present in the

p38Kb$^{\Delta 45}$ mutants including the pellet (Figure 7B), suggesting that loss of p38Kb

prevents the effective clearance of these Lamin cleavage forms. Interestingly, loss of the

Lamin CaaX box does not affect the formation of these lower molecular weight forms,

however, it does result in an increase in the phosphorylation of full-length Lamin (Figure

7D). Additionally, we do not detect phosphorylation positive high molecular weight forms

of Lamin (Figure 7D), suggesting that farnesylated Lamin is not phosphorylated at

Ser45. Overall these data suggest that Lamin is processed in a variety of different ways

in the adult muscle and these different forms are prone to aggregation in the absence of

p38Kb.

To determine if Lamin Dm_0 is a direct target of p38Kb and the CASA complex, we

tested if Lamin Dm_0 can bind to the CASA complex. We immunoprecipitated

endogenously GFP tagged HspB8, Hsc70-4 and stv from adult *Drosophila* muscle and

probed for endogenous Lamin Dm_0. We find that full-length Lamin Dm_0 co-

immunoprecipitates with all three members of the CASA complex (Figure 3G and Figure

S1E), but do not detect binding with either the higher or lower molecular weight forms of

Lamin Dm_0 found in the fractions (Figure 7A and B). Furthermore, we expressed Lamin

Dm_0 tagged with GFP in the muscle and found that endogenous stv co-

immunoprecipitates with Lamin Dm_0 (Figure 3G and Figure S1F). These data suggest

that the CASA complex is able to bind to full-length Lamin Dm_0 for targeting to the

autophagosome.

As we hypothesize that p38Kb is interacting with the CASA complex at the point

in which the poly-ubiquitinated misfolded targets are handed over to ref(2)p for

degradation, we tested if p38Kb also binds to Lamin Dm_0. We find that p38Kb^{KD}

expressed in the muscle co-immunoprecipitates with both high and low molecular weight

forms of Lamin Dm_0 (Figure 6G). Interestingly, p38Kb physically interacts with the

aggregate prone low molecular weight species of Lamin Dm_0 as opposed to the

predominant full-length form of Lamin Dm_0 (Figure 6G). More striking is that p38Kb^{KD}

physically interacts with increasingly larger forms of Lamin Dm_0 (Figure 6G), suggesting

that p38Kb is interacting with the poly-ubiquitinated forms of Lamin Dm_0. Furthermore,

we have expressed GFP-tagged Lamin Dm_0 in the muscle and were able to pull down

phosphorylated p38K (Figure 3G). These data indicate that p38Kb activation may be

important for the hand off of misfolded proteins that are tagged for lysosomal

degradation from stv to ref(2)p.

We have developed a model in which misfolded proteins such as the main form

of Lamin Dm_0 are targeted by stv to the CASA complex. Those proteins that cannot be

refolded are then tagged with poly-ubiquitin to signal their degradation through the

autophagosome/lysosome pathway. These poly-ubiquitinated proteins are handed off by

stv to ref(2)p in a process mediated by activated p38Kb. We hypothesize that this hand

off is a rapid process as we were unable to detect the higher molecular weight poly-

ubiquitinated forms binding to the CASA complex and were only able to capture these

transient interactions using the p38Kb[KD] construct. This model explains our finding that

in p38Kb mutants these poly-ubiquitinated proteins accumulate and then form

aggregates, and how over-expression of p38Kb would lead to increased efficiency of the

poly-ubiquitinated proteins being targeted to the autophagosome. In agreement with our

model, full-length Lamin Dm_0 would bind to the CASA complex for refolding and release,

or for poly-ubiquitination and transfer to ref(2)p for degradation through an interaction

with p38Kb.

Discussion

We find that the aging gene p38Kb regulates age-dependent protein homeostasis

through an interaction with the CASA complex and is acting at a step between the poly-

ubiquitination of an un-foldable target and its transfer to ref(2)p for autolysosomal

mediated degradation. Interestingly, we find that p38Kb is important for the proper

localization of stv to the Z-disc but does not affect the localization of other complex

members. This suggests that p38Kb may play a role in maintaining the interaction

between stv and the CASA complex which may be necessary for target transfer to

ref(2)p. stv has a conserved MAPK docking site as well as eight potential p38K

phosphorylation sites. Furthermore, the mammalian ref(2)p homolog p62 has been

shown in vitro to bind to mammalian p38K through two domains [74], which are partially

conserved in flies. Therefore, one possibility is that p38Kb mediated phosphorylation of

stv facilitates the localization of stv to form a functional CASA complex at the Z-disc,

where damaged proteins are rapidly turned over. Another possibility is that p38Kb binds

to stv and ref(2)p, and that the phosphorylation of stv is required for target hand-over to

ref(2)p so that in the absence of p38Kb, stv cannot transfer targets to ref(2)p. A

consequence of this may be that stv and the protein target together are released from

the CASA complex, leading to mislocalization of stv. As we don't detect a decrease in

phospho-Lamin in the p38Kb mutants or find that the p38Kb can pull-down phospho-

Lamin, it is unlikely that p38Kb is directly phosphorylating the target proteins as a part of

the stv-ref(2)p hand-over process.

How protein aggregation contributes to aging and disease has been an area of

great interest. One outstanding question is if protein aggregation is a consequence or

cause of aging. It has been hypothesized that protein aggregates accumulate with age

as the amount of damaged or misfolded proteins increase. However, it is not clear

whether or not these aggregating proteins are toxic leading to tissue dysfunction and a

disease state. Previous studies have found that long-lived fly strains such as over-

expression of Foxo or parkin result in reduced protein aggregate formation [4,75].

Therefore, we hypothesized that decreased protein aggregation would lead to a lifespan

extension, while increased protein aggregation would lead to a reduced lifespan. As

expected, we find that the short-lived p38Kb mutants, which exhibit premature locomotor

behavior defects[16], have large and numerous protein aggregates. We also find that over-

expression of p38Kb leads to decreased protein aggregation and increased lifespan.

However, while inhibition of stv results in increased aggregation and decreased

lifespan, over-expression of stv leads to decreased aggregate number without a

concurrent lifespan extension. These data suggest that protein aggregation and lifespan

may be separable processes. The CASA complex is important for maintaining lifespan

as inhibition of stv results in a shorter lifespan. However, as stv over-expression on its

own doesn't extend lifespan, this suggests that lifespan extension may not entirely

depend on the CASA complex or that increasing the activity of the CASA complex alone

is not sufficient to drive lifespan. It has been hypothesized that the CASA complex is

selective and only targets a subset of proteins for refolding/degradation. This selectivity

is provided by BAG-3 in mammals and presumably stv in flies, though the selectivity of

the complex has not been fully characterized in flies. This selectivity may explain why

the CASA complex alone does not extend lifespan, as it may not target proteins that are

known to cause increased lifespan but rather targets proteins that are involved in

maintaining organismal health/lifespan.

Another potential interpretation of these results is that aggregate number is not

as critical as aggregate size and/or content, particularly early in life, for lifespan

extension. We find that stv over-expression only results in decreased aggregate size in

older animals, however, co-over-expression of p38Kb and stv leads to reduced

aggregate size at both young and old ages and leads to a further increase in lifespan.

Thus, the turnover of specific toxic protein or subset of proteins early in life may lead to a

reduction in the exposure to these toxic species and a lifespan extension. Furthermore, it

has been hypothesized that protein aggregates may be protective in some instances.

For example in Alzheimer's disease it has been hypothesized that the soluble form of

Amyloid-β is toxic and that the formation of aggregates protects against this toxicity [76–

[80]. If aging or lifespan is dictated by the presence of soluble toxic proteins, then reducing

these toxic proteins would increase lifespan. As over-expression of stv is unable to

extend lifespan, this would then suggest that these particular soluble toxic proteins are

not targets of the CASA complex.

Our data also suggest that the p38Kb can mediate lifespan through additional,

CASA complex-independent mechanisms. Indeed, we have previously shown that

p38Kb regulates lifespan at least in part by regulating oxidative stress[16]. Additionally, we

find that over-expression of Hsc70-4, which is involved multiple protein quality control

pathways, is able to extend life span, supporting the idea that CASA alone may not be

sufficient to clear out all the toxic protein species that play a role in aging.

It has recently been found that over-expression of the related Hsp70 Ab gene in

Drosophila adult muscle does not extend lifespan[81]. One possibility as to why we

observe a lifespan extension is that we over-expressed Hsc70-4 using a different GAL4

driver (Mef2-GAL4 as compared to DJ694-GAL4) that drives expression all through

development and in the adult though expression in the adult declines with age (Figure

S2D). It may be that this early over-expression of Hsc70-4 provides a beneficial effect

that protects the fly from general life stress, resulting in increased longevity. Another

possibility is that Hsc70-4 and Hsp70 Ab proteins, which are 89.6% similar and 73%

identical, have different cellular specificities that give rise to different functions resulting

in different lifespan responses.

We find that Lamin, which is mutated to an aggregation prone protein form in

Hutchinson-Gilford progeria [46,55,57,72,82], is a target of p38Kb and the CASA complex.

Interestingly, p38Kb and Lamin mutants share similar phenotypes such as age-

dependent locomotor impairments [16,64,65] and upregulation of the Nrf-2/Keap-1 pathway

[16,51]. As loss of p38Kb leads to an accumulation of Lamin in the aggregates, this may be

toxic to the cell, leading to impaired locomotor function, increased stress and decreased

lifespan. Thus, we have found that one aging gene (p38Kb) regulates a second,

unrelated aging gene (Lamin) via the CASA complex. These data suggest a new link

between aging pathways and how they may converge through the regulation of protein

homeostasis.

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Figure Legends

Fig 1. p38Kb regulates age-dependent protein homeostasis.

Confocal micrographs of polyubiquitin positive protein aggregates in the adult indirect

flight muscle in **A)** a precise excision genetic background control p38Kb$^{Ex41/Ex41}$ and **B)**

p38Kb$^{\Delta45/\Delta45}$ null mutants at three weeks of age and in **C)** outcrossed Mef2-GAL4

controls (Mef2>w^{11118}) and **D)** UAS-p38Kb^{wt} Mef2-GAL4 (Mef2>p38Kb^{wt}) over-expression

animals at 5 weeks of age. Scale bar equals 6.2 μm. Box-Whisker plots of aggregate

number in p38Kb$^{\Delta45/\Delta45}$ mutants as compared to p38Kb$^{Ex41/Ex41}$ controls at **E)** 1 week and

F) 3 weeks of age and aggregate size at **G)** 1 week and **H)** 3 weeks of age. Aggregate

number in p38Kb^{KD} Mef2-GAL4 (Mef2>p38Kb^{KD}) and outcrossed Mef2-GAL4 controls at

I) 1 week and **J)** 5 weeks of age and aggregate size at **K)** 1 week and **L)** 5 weeks of

age. Aggregate number in strong p38Kb over-expression animals and outcrossed Mef2-

GAL4 controls at **M)** 1 week and **N)** 5 weeks of age and aggregate size at **O)** 1 week

and **P)** 5 weeks of age. Aggregate number in moderate p38Kb over-expression

(MHC>p38Kb^{wt}) animals and outcrossed MHC-GAL4 controls (MHC>w^{1118}) at **Q)** 1 week

and **R)** 5 weeks of age and aggregate size at **S)** 1 week and **T)** 5 weeks of age.

Fig 2. p38Kb regulates aging phenotypes through ref(2)p.

A-C) A subset of polyubiquitinated protein aggregates (green) in 3 week old

p38Kb$^{Ex4/Ex41}$ control muscle contain K63 ubiquitinated (magenta) proteins (white

arrows). **D-F)** K63 ubiquitin positive protein aggregates are also observed in 3 week old

p38Kb$^{\Delta45/\Delta45}$ mutant muscle. Protein aggregate number in ref(2)p heterozygous mutant

backgrounds at **G)** 1 week and **H)** 5 weeks. Loss of a single copy of ref(2)p prevents the

p38Kb mediated reduced protein aggregation at 1 week and 5 weeks of age. Asterisks

denote a p-value of ≤0.001.

Fig 3. p38Kb genetically interacts with stv to regulate protein homeostasis and

lifespan (A-D) Localization of a FLAG-tagged p38Kb (green in **A-D** and **A''-D''**) in the

adult indirect flight muscle. **A)** FLAG-tagged p38Kb localizes to the Z-disc (arrows) as

exhibited by colocalization with the Z-disc protein alpha-actinin (magenta, **A'** and **A''**), as

well as the M-line (arrowheads). **B-D)** p38Kb colocalizes with each CASA complex

member (magenta, **B'-D'**) at the Z-disc. Over-expression of a FLAG tagged p38Kb in the

muscle in **B**) an endogenous Hsc70-GFP fusion protein background, **C**) an endogenous

HspB8-GFP fusion protein background, and **D)** a wildtype background with endogenous

levels of stv. **E-F)** Muscle lysates expressing control and Mef2-GAL4 UAS-p38Kb$^{KD-FLAG}$

in either **E)** an endogenously expressed HspB8-GFP background or **F)** a wildtype

background were immunoprecipitated using anti-FLAG coated beads. **E)** Immunoblots

were probed with anti-GFP to detect the presence of HspB8 in the IP lysates or with **F)**

anti-stv to detect the presence of stv in the IP lysates. **G)** Muscle lysates expressing

control, UAS-mito-GFP, endogenously tagged stv or HspB8, or over-expressing MHC-

GAL4 UAS-Lamin^{GFP} were immunoprecipitated using anti-GFP coated beads.

Endogenous Lamin (~75kDa) was pulled down by stv-GFP. In addition, Lamin-GFP

(~100kDa) was also detected as a control. Endogenous phoshop-p38K was pulled down

by both stv and lamin. Endogenous stv (~80kDa) was pulled down by both HspB8 and

Lamin. In addition, as a control we can detect pull down of the stv-GFP (~100kDa). Anti-

GFP was used to confirm to the pull down of both GFP alone and HspB8-GFP.

Fig 4. p38Kb genetically interacts with stv to regulate protein homeostasis and

lifespan. A) Protein aggregate number and **B)** size in the moderate stv knockdown

background using MHC-GAL4 at 5 weeks. Asterisks denote a p-value of ≤0.001. **C)**

Moderate over-expression of p38Kb (red line) results in an increased lifespan as

compared to the MHC-GAL4 controls and p38Kb transgene control (black line and gray

lines, respectively). Moderate knockdown of stv using the MHC-GAL4 results in a

decreased lifespan (pink line compared to yellow and black lines) and is rescued by

p38Kb over-expression (compare pink line to blue line). **D)** Strong over-expression of

p38Kb (red line) results in an increased lifespan as compared to the Mef2-GAL4 controls

and p38Kb transgene control (black line and gray lines, respectively). Strong knockdown

of stv using the Mef2-GAL4 results in a decreased lifespan (pink line compared to yellow

and black lines) and is partially rescued by p38Kb over-expression (compare pink line to

blue line). **E)** Over-expression of stv in the p38Kb mutant background results in a further

reduction of lifespan as compared to p38Kb mutant controls (compare blue line to red

and grey lines).

Fig 5. p38Kb is limiting for stv function and localization.

Protein aggregate number at **A)** 1 week and **B)** 5 weeks and protein aggregate size at

C) 1 week and **D)** 5 weeks measured in stv over-expression backgrounds. Over-

expression of stv leads to reduced aggregate number at 1 and 5 weeks and aggregate

size at 5 weeks. Co-over-expression of stv and p38Kb does not result in a further

decrease in protein aggregate number but trends towards decreased aggregate size at

both 1 and 5 weeks of age. Asterisks denote a p-value of ≤0.001 when compared to the

GAL4 control. **E)** Over-expression of stv alone has no significant effect on lifespan (pink

line as compared to yellow and black lines), however, co-over-expression of stv and

p38Kb results in a further increase in lifespan (compare red line to blue line). **F)**

Immunoblots of stv from 1 and 3 week old muscle lysates of control and p38Kb mutants.

G) stv localizes to the adult muscle Z-disc and M-line (white arrows) in control animals.

H) stv localization is disrupted in p38Kb mutants. **I)** Quantification of average pixel

density.

Fig 6. p38Kb and stv regulate Lamin aggregation.

A) Immunoblot analysis of stv-RNAi and GAL4 controls flies muscle lysates probed with

anti-Lamin and **B)** quantified using densitometry, asterisk denotes p value=0.028. **C)**

Immunoblot analysis of p38Kb$^{Ex41/Ex41}$ control and p38Kb$^{Δ45/Δ45}$ mutant muscle lysates

probed with anti-Lamin and **D)** quantified using densitometry, asterisk denotes p value =

0.006643. **E)** Immunoblot analysis of p38Kb$^{Ex41/Ex41}$ control and p38Kb$^{Δ45/Δ45}$ mutant

muscle lysates probed with anti-phospho Lamin and **F)** quantified using densitometry,

p=0.09. **G)** p38Kb^{KD} was expressed in muscles using Mef2-GAL4. p38Kb was

immunoprecipitated from adult muscle lysates using anti-FLAG coated beads.

Immunoblots were probed with anti-lamin and anti-FLAG.

Fig 7. Lamin aggregation is increased in aged p38Kb mutants.

A-C) Immunoblots of sucrose gradient fractions from 3 week old p38Kb$^{Ex41/Ex41}$ control

and p38Kb$^{Δ45/Δ45}$ mutant muscle. **A)** The main species of Lamin (~75kDa) is mainly

found in fractions 5-7 with some in the pellet, and the farnesylated form of Lamin

(~100kDa) is mostly found in the pellet in controls. In the p38Kb mutants, there is a

further accumulation of Lamin and farnesylated Lamin in the pellet. **B)** Phosphorylated

Lamin is also found predominantly in fractions 5-7 in controls and accumulates in the

pellet in p38Kb mutants. **C)** Poly-ubiquitin is found mostly in the pellet fraction, marking

the protein aggregates. **D)** Immunoblots of control and Lamin^{A25} mutants that lack the

CAAX motif demonstrate the loss of the farnesylated form of Lamin (100kDa) without

affecting the main form of Lamin (~75kDa). In addition, phosphorylated Lamin is also

present in the Lamin mutants.

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Figure 1

Figure 2

	p38Kb	CASA Member	Merge
α-actinin	A	A'	A''
Hsc70	B	B'	B''
HspB8	C	C'	C''
stv	D	D'	D''

E

IP:anti-FLAG

Mef2>p38Kb^{KD}-FLAG	−	+
HspB8-GFP	+	+
IB:anti-GFP		
IB:anti-FLAG		

F

Mef2>p38Kb^{KD}-FLAG	−	+
IB:anti-stv		
IB:anti-FLAG		

G

IP:anti-GFP

w^{1118} GFP stv-GFP HspB8-GFP Lam-GFP

IB:anti-Lamin
- 250 kDa
- 150 kDa
- 100 kDa
- 75 kDa

IB:anti-p-p38K
- 50 kDa
- 37 kDa

IB: anti-stv
- 150 kDa
- 100 kDa
- 75 kDa

IB: anti-GFP
- 50 kDa
- 37 kDa
- 25 kDa

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure S1