Aggregation of PolyQ Proteins Is Increased upon Yeast Aging and Affected by Sir2 and Hsf1: Novel Quantitative Biochemical and Microscopic Assays

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Introduction

Huntington’s disease (HD) is a progressive neurodegenerative disorder manifested by dysfunction and cell death mainly in the striatal and cortical brain regions [1–3]. HD patients experience deteriorating symptoms including chorea, progressive dementia, psychiatric manifestations, and death within 15 years of symptoms onset. Currently there is neither cure nor treatment to postpone the onset of HD or to slow its progression [4–6]. HD is an autosomal dominant genetic disease, caused by mutations in the first exon of the huntingtin gene, Htt, that increase the number of CAG repeats to generate expanded polyglutamine (polyQ) tracts in the N-terminal region of the huntingtin protein [7].

It is still unclear why the ubiquitously expressed huntingtin has a distinctive neurological phenotype and if huntingtin’s loss-of-function leads to HD [8;9]. The widely accepted notion is that HD is caused by a harmful gain-of-function of expanded polyQ since mice models with mutated huntingtin exon 1 recapitulate HD symptoms, including neuronal dysfunction and death [10]. Hence, although CAG repeat RNA was recently proposed as an auxiliary toxic agent in polyQ disorders [11], it appears that the deleterious effects in HD are mostly related to the tendency of the mutated huntingtin protein to misfold and aggregate. This property is observed with purified proteins and in neurons, where cellular aggregates are mainly composed of huntingtin [12]. In vivo and in vitro studies of polyQ fibrillogenesis indicate that aggregation is an intrinsic feature of long polyQ tracts and this tendency increases with the length of the polyQ repeat [13;14]. Indeed, HD is one of nine known polyQ repeat autosomal dominant disorders, all of which result from mutations that expand the polyQ tract within different but specific proteins [15–18]. Although the mutated proteins have no shared qualities beside the polyQ mutation, all these disorders are characterized by progressive neurodegeneration as well as the formation of polyQ protein aggregates.

The common risk factor of all aggregation diseases is aging [19–21] and, in HD, the combination of aging and genetic mutations is manifested by inverse correlation between the age of disease onset and the number of CAG repeats. Repeats shorter than 35Q are asymptomatic, whereas repeats exceeding the 40Q threshold ensure HD development such that 40–50Q give rise to the normal adult-onset of HD while longer repeats result in juvenile cases [22–27]. Although various statistical models strongly correlate the mean age of HD onset with the length of the CAG repeat [28;29], patients with the same polyQ length exhibit very different ages of onset. Hence, modifiers of HD and aggregation, apart from the length of the polyQ, must be implicated [5]. Based on screening HD patients or model organisms, combinations of genetic and environmental factors clearly affect the age of HD onset [22].

Cellular aging is considered as a progressive decline in the proteostasis machinery and the response to changing environment.
Aging is caused partly by genetic factors accompanied by metabolic, environmental and stochastic factors [30;31]. Progress in cellular aging research is driven by single-celled eukaryotes such as the budding yeast *Saccharomyces cerevisiae*. In this organism, genetic modulators of replicative lifespan (RLS) are being identified, and chronological lifespan (CLS) is considered useful for understanding the aging process in non-dividing mammalian cells such as neurons [32;33]. Importantly, a subset of pathways that influence longevity in yeast is conserved in other eukaryotes, including mammals. A genome-wide screen in *S. cerevisiae* has identified CLS-affecting genes that are highly conserved in other species, suggesting that longevity is a fundamental process conserved in evolution [34]. Finally, the stationary phase model of aging in yeast recapitulates many pathological alterations observed during neuronal aging, highlighting the power of yeast as a model system to explore the molecular basis of aging-related diseases of the central nervous system [35].

Among the conserved genes implicated in determining lifespan are the siruins, a family of class III NAD+-dependent protein deacetylases. In their unique lysine deacetylation reaction, NAD+ is cleaved, 29-O-acetyl-ADP-ribose is generated and nicotinamide (NAM) is released. Silent information regulator 2 (Sir2) in yeast or its closest mammalian homolog Sirt1 are conserved from yeast to mammals and are shown to regulate metabolism and longevity [36–38]. RLS in yeast is extended when Sir2 is overexpressed and decreases upon SIR2 deletion [39]. Also, lifespan extension associated with dietary restriction (DR) requires Sir2 and NAD+ since it is nullified by deletion of either SIR2 or NPT1 (a gene in the NAD+ biosynthetic pathway). However, the role of siruins in DR-mediated lifespan extension is controversial; in yeast at stationary phase, Sir2 actually blocks extreme CLS extension mediated by DR [40].

Heat shock factor 1 (Hsf1), a master regulator of transcription, is another highly conserved protein that plays an important role in longevity as well as in maintaining proteostasis and adequate response to proteotoxic stresses. In *Caenorhabditis elegans*, Hsf1 is required for enhanced thermotolerance and suppression of proteotoxicity and was also shown to regulate aging [41;42] and to affect lifespan extension by DR [43]. Hsf1 is a regulator of lifespan also in yeast, as the CLS extender Ec2 in *Schizosaccharomyces pombe* and its functional homolog YGR146C in *S. cerevisiae* are direct targets of Hsf1 and overexpression of Hsf1 in fission yeast extends CLS [44]. Hsf1 is activated by heat-shock, oxidative, metabolic or environmental stresses, although our understanding of Hsf1 regulation remains incomplete. In yeast, under normal growth conditions, Hsf1 is a constitutively phosphorylated homotrimer but inactive. Upon exposure to stress, Hsf1 is hyperphosphorylated and adopts an active conformation. Hsf1 binds to heat shock response elements (HSE) in the promoter region of its target genes and activates their expression [45–48].

Among the many Hsf1 target genes are the molecular chaperones, which resolve damaged/misfolded and aggregated proteins generated by heat stress or metabolism [49;50]. Interrelations between Hsf1 and Sir1, the closest mammalian homolog of Sir2, may underlie their effects on proteostasis. Sir1 phosphorylation is required for cell survival under stress, and mammalian Hsf1 is one of the substrates that phosphorylated Sir1 deacetylates and co-activates, allowing the deacetylated Hsf1 to bind to HSE and activate the molecular chaperones network [51–53].

Among the genetically manipulable model organisms for studying aggregation and toxicity of polyQ proteins, *S. cerevisiae* provides unsurpassed tools to decipher disease-associated cellular processes and identify novel therapeutic targets [54]. Although this unicellular eukaryote neither resembles neurons nor expresses endogenous huntingtin, the relevant cellular pathways appear to be highly conserved between humans and yeast. Hence, yeast is an established system for studying the causes and consequences of polyQ aggregation, and to address aging, DR and oxidative stress at the cellular and molecular levels [33;35;55–67]. Here we follow the effects of aging and altered expression or activity of SIR2 and HSF1 on the aggregation of polyQ proteins in *S. cerevisiae*. Our most interesting results are with the 47Q that harbors a mid-size polyQ tract characteristic of the threshold of HD onset. We find that 47Q is non-toxic and soluble in growing yeast, but aggregates as cells age. Upon SIR2 deletion, the aging-dependent aggregation is aggravated, while overexpression of Hsf1 attenuates aggregation. Hence, Sir2 and Hsf1 appear to affect aggregation-related processes during aging.

**Results**

**Quantitative measurements of polyQ proteins aggregation in yeast cells**

Aggregates of polyQ proteins and their distribution in live cells are usually detected by fluorescence microscopy, owing to a fluorescent tag attached to the polyQ proteins [55;56]. Although aggregation patterns differ from cell to cell, images are usually evaluated qualitatively and any quantitative analysis merely evaluates qualitatively and any quantitative analysis merely

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Increased aggregation of long and mid-size polyQ protein is observed upon aging

The quantitative analysis allows us to monitor the aggregation of the various polyQ proteins during cell aging and draw a timeline for the aggregation process. Aggregate formation of the long 103Q occurs very early and it somewhat increases as the cells age, while the short 25Q does not aggregate at any age (Figure 3B, C). Importantly, the mid-size 47Q does not aggregate in logarithmically growing young cells, but its aggregation is markedly increased as the cells age (Figure 3B, C). The 47Q aggregation starts when the cells enter the stationary phase and stop dividing and progresses with age (Figure 3C).

This aggregation of the various polyQ proteins and especially the aging-dependent change in 47Q aggregation is strongly corroborated by our quantitative fluorescence microscopy. To quantify the aggregation level within individual cells, we analyze in hundreds of cells per sample the ratio $R$ between the GFP fluorescence in the brightest focus and the average GFP fluorescence in the whole cell (Figure 4 A, B and Figures S1, S2 and S3). In 25Q-expressing cells with no aggregates this ratio is close to 1 (Figures 4B and S1), 103Q-expressing cells with aggregates show ratio values higher than 1 (Figures 4B and S3), and in 47Q-expressing cells the ratio increases upon aging (Figures 4B and S2). When $R$ is plotted along the yeast aging process (Figure 4C), this ratio is in striking agreement with the Aggregation Index determined by filtration (Figure 3C). By defining cells with $R$ above a cutoff of 1.5 as having aggregates, we calculated the fraction of cells with aggregates (Figure 4D). Both quantitative approaches reflect the aging-dependent aggregation of the 47Q, with no aggregation of the 25Q and immediate onset of aggregation of the 103Q. While this manuscript was under revision, a study in C. elegans reported the in vivo dynamics of polyQ aggregation using fluorescence microscopy, fluorescence recovery after photobleaching and fluorescence correlation spectroscopy [68]. Also in that study, the brightest foci represented aggregates, as they were immobile. The decline in $R$ of 103Q at late time points stems from an age-dependant decline of total GFP fluorescence in these cells, as shown in Figures 4B and S3.
this decline is not observed in the filter retardation assay (Figure 3),
it is likely not the consequence of aggregates loss. Combined, our
two quantitative analyses indicate that the mid-size 47Q is soluble
in growing yeast and aggregates progressively as the yeast age.

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aggravated, while overexpression of Hsf1 attenuates aggregation

One of the proteins that are firmly linked to aging is the NAD+-
dependent protein deacetylase Sir2. Thus, we next followed the
aggregation of polyQ proteins in a mutant lacking the SIR2 gene.
When compared to wild-type cells (Figure 3C), it is evident that
despite similar expression level of the polyQ proteins, the
aggregation of both 47Q and 103Q is increased remarkably in
aging Δsir2 cells (Figure 5A, squares and triangles, respectively),
and irrespective of SIR2 deletion, 25Q still does not aggregate
(Figure 5A, diamonds). Being a class III deacetylase that cleaves
NAD⁺ for its activity, Sir2 releases nicotinamide (NAM), which is
reported to be a noncompetitive inhibitor of Sir2 [69]. Indeed, in
the presence of NAM, 47Q aggregation increases in aged cells
(Figure 5B) in a concentration-dependent manner (Figure 5C).
Even the highest concentration of NAM (40 mM) affects neither
the already highly aggregated 103Q nor the never aggregated 25Q
(Figure 5C).

Another aging-related protein, whose activity and content are
critical for the cellular response to proteotoxic stresses, is Hsf1
[41;43;44]. When HSF1 is overexpressed from a plasmid in wild-
type cells, we find attenuated aging-dependent aggregation of the
mid-size 47Q and the long 103Q, as compared to cells expressing
an empty plasmid (pRS314) instead of pHSF1 (Figure 5D). Our
results indicate that Hsf1 is involved in aggregation of polyQ
proteins in aging cells.
triplicates each, are presented as mean proteins were quantified and Aggregation Index C was calculated as described in and in Figure 2. Data from 3 independent transformants, 3 Figure 2) were lysed. Early time points (8–16 hrs) were done separately from later time points. Total amounts A and captured aggregates B of polyQ p 25Q, 47Q or 103Q were grown under galactose induction for the indicated time, and at each time point 11.25

Discussion

Neurodegeneration and aging, intimately interrelated processes, are the subject of intensive clinical research. The complexity of both processes, let alone when combined, makes it extremely difficult to elucidate the mechanisms that underlie aging-dependent neurodegeneration. Aging is poorly defined at the molecular level and the effects of aging on cellular processes are not fully understood. Nevertheless, many key genes implicated in longevity are highly conserved in evolution, suggesting that they operate in fundamental mechanisms. Neurodegenerative diseases are also diverse but one of their major hallmarks is the deposition of specific aggregation-prone proteins. To date neither the basis for the toxicity of these aggregates, nor their formation or the cellular processes affected by them is fully explained. Because of the close links between the two processes, models in mice, flies, worms and organisms, including yeast where it was first discovered, is the class-III deacetylase Sir2, although its role in aging remains controversial. As noted above, there are two models of aging in yeasts, RLS and CLS [75]. Whereas replicative aging may be a useful model for mitotically active cells, chronological aging represents more faithfully postmitotic cells, such as neurons. Interestingly, Sir2 is beneficial for RLS, whereas Δsir2 cells show higher CLS and better resistance to different stress conditions [40]. Here we find in aging Δsir2 cells increased aggregation (Figure 5A) that is also detected when Sir2 activity is blocked by NAM (Figure 5B, C).

Although further investigation is required to understand the role of Sir2 in aggregates formation in aging yeast, this phenomenon

suggested that our results with the near-threshold mid-size 47Q in aging yeast closely simulate the situation in humans. Thus, aging yeast may serve a reliable model system to study the dependence of aggregation on aging, a hallmark of many neurodegenerative disorders.

We show that yeast aging affects the aggregation of polyQ proteins and implicate aging-related genes in this phenomenon (Figures 3,4,5). Clearly, HSF1 overexpression decreases aggregation of 47Q or 103Q (Figure 5D). These results may reflect an aging-dependent functional decline in Hsf1 and/or its many targets, mostly the chaperone network. Indeed, upregulation of chaperones increases longevity and enhances aging-related stress resistance in various models [70]. In particular, overexpression of Hsp70 extends the lifespan of C. elegans [71] and Drosophila melanogaster [72]. Likewise, a very recent screen of molecules has identified new classes of proteostasis regulators that induce HSF–1–dependent chaperone expression and restore protein folding in multiple conformational disease models [73]. It appears that proteostasis diseases are aggravated when the capacity of the chaperone network to cope with inherited misfolding-prone proteins, aging, or metabolic/environmental stresses declines [74].

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Figure 4. Quantitative microscopic assay reveals that aggregation of 47Q increases upon aging. A Wild-type cells (W303–1b) expressing 25Q, 47Q or 103Q were grown under galactose induction for up to 42 hrs. At each time point hundreds of cells were imaged by fluorescence microscopy. Images of 3 representative time points are presented. B Microscopic images from a similar experiment were analyzed for the presence of aggregates in individual cells as presented by the ratio between the maximal density (y axis) and the density (x axis) and 3 representative time points are presented (for all time points, see Figures S1, S1 and S3). C The ratio $R$ between the maximal density and the density throughout the experiment plotted over time. At each time point the mean values ± SD are shown and the error bars reflect the variability between individual cells. D The fraction of cells with aggregates (defined as cells with $R$ above a cutoff of 1.5) plotted over time.

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may reflect accelerated aging and failure to segregate damaged proteins in Sir2 mutants [76], possibly due to Sir2 involvement in maintaining the activity of the TRiC/CCT complex [77]. The ongoing debate of whether aggregates are protective or harmful to cells is still open. Uncoupling between aggregation and toxicity was recently reported from RNA interference genetic screens in C. elegans that have identified novel regulators of the proteostasis network [78]. Indeed, aggregates are observed in affected cells and serve as a hallmark of Alzheimer’s, Parkinson’s and HD. Also, in HD, polyQ aggregates are detected before the onset of clinical symptoms [79]. Yet, the formation of polyQ aggregates was reported to improve neurons survival compared to cells that had more diffuse distribution of huntingtin [80]. Moreover, analysis of polyQ structural changes showed that a soluble monomeric beta-sheet conformer of the expanded polyQ was a major cause of cytotoxicity [81]. Therefore, large aggregates may be the result of an active mechanism that has evolved to protect the cell by sequestering the toxic species, either the misfolded proteins and/or their oligomers [82;83]. It remains to be established how Sir2 and Hsf1 affect, directly or indirectly, the aggregation of polyQ proteins. Interestingly, deacetylation by Sirt1 (the closest mammalian homolog of Sir2) is reported to be one of the activation modes of the mammalian Hsf1 [51]. If yeast Hsf1 activity is also regulated by Sir2, this suggests a coordinated mechanism designed to handle aggregation-prone proteins.

The filter retardation aggregation assay we describe here (Figure 2) is simple, reproducible, and linear within a wide range of concentrations. Supported by the fluorescence microscopy (Figure 4), these assays allow monitoring the level of protein aggregation throughout the life of yeast populations in a quantitative fashion. Both assays can be easily applied to other cells and organisms. By these methods, and capitalizing on the ease and speed of genetic screening in yeast, this model system is particularly amenable to study the effects of genetic and environmental factors on aging and aging-dependent aggregation and determine their consequences on cytotoxicity and cell survival.

**Materials and Methods**

**Yeast strains and plasmids**

The yeast strains of Saccharomyces cerevisiae employed in this study are W303–1b (MATa ura3–52 tpiD2 leu2–3,112 his3–11 ade2–1 can1–100) and KFY100 (MATa his4–619 leu2–3,112 ura3–52). Δsir2 (RS1717; W303–1b sir2Δ::his5) was generously provided by

Figure 5. Sir2 and Hsf1 are involved in the aging-dependent aggregation of polyQ proteins. A Δsir2 (RS1717; W303–1b sir2Δ::his5) cells expressing 25Q, 47Q or 103Q were grown under galactose induction for the indicated time, collected, lysed, and Aggregation Index was calculated for each time point, as described in Figure 3. Data from 3 independent transformants, 3 triplicates each, are presented as mean ± SE. B Wild-type (WT, W303–1b) or Δsir2 cells expressing 47Q were grown for the indicated time under galactose induction. Wild-type cells were also exposed to 10mM nicotinamide (NAM). Cells were collected, lysed and Aggregation Index for each time point was calculated as described in Figure 3. C Wild-type cells (W303–1b) expressing the indicated polyQ proteins were grown under galactose induction for 72 hours with the indicated concentration (mM) of NAM. Cells were collected, lysed and Aggregation Index was calculated as described in Figure 3. D Wild-type (W303–1b) cells expressing the indicated polyQ proteins and harboring either an empty (pRS314) or pHSF1 plasmid were grown under galactose induction for the indicated time. Cells were collected, lysed and Aggregation Index was calculated for each time point as described in Figure 3. Data from 3 independent transformants, 3 triplicates each, are presented as mean ± SE.
containing pYES2 vector, so the regulated expression of the URA3 (Figure 1A). The constructs were subcloned into theMinimal liquid media, starting at 0.05 A600 and following theirgrowth up to 0.8 A600, for calculating the duplication time.

Flag-Htt17-Q47-gfp (47Q) and Flag-Htt17-Q103-gfp (103Q)(GFP) at the C-terminus, generating Flag-Htt17-Q25-gfp (25Q),minimal liquid media, the appropriate nutrients required forselection, and 2% (w/v) glucose or 4% (w/v) galactose. Alternativa-yeast nitrogen base, the appropriate nutrients required for

Growth conditions

Yeast were regularly grown in minimal media which contained0.67% (w/v) yeast nitrogen base, the appropriate nutrientsrequired for selection of transformants, and 2% (w/v) glucosel. For galactose induction, cells were transferred to mediumcontaining 4% (w/v) galactose only, or 2% (w/v) galactose and2% (w/v) fructose. Stationary phase cells that stopped dividing andreached 3.0-6.0 A600 (starting from 1.5 x 10^6 cells/ml of glaucose starters; cell number was calculated as A_{600} = 1.5 x 10^6 cells/ml)were grown for up to 15 days. All cells undergoing galactoseinduction were grown in 10 ml medium in 100 ml loosely-cappedbottles, unless indicated otherwise.

For growth experiments, cells were grown logarithmically (0.6-1 A_{600}) diluted to equal cell density (0.5 A_{600}) and spotted on 10-fold serial dilutions on plates containing 2% (w/v) agar, 0.67% (w/v) yeast nitrogen base, the appropriate nutrients required forselection, and 2% (w/v) glucose or 4% (w/v) galactose. Alterna-tively, yeast cells were grown in glucose- or galactose-containingminimal liquid media, starting at 0.05 A_{600} and following theirgrowth up to 0.8 A_{600}, for calculating the duplication time.

Quantitative fluorescence microscopy

Yeast grown to logarithmic or stationary phase were viewedunder the DMRE fluorescence microscope (Leica). GFP wasviewed using Chroma 41017 filter (excitation 470/40) and snapshots were taken using Magnafire 12-bit color CCD camera(Figure 4A). For aggregate quantification (Figure 4B), yeas grown tologarithmic or stationary phase were imaged on a Nikon TiEfluorescence microscope with a 100X/1.49NA objective. GFP images of hundreds of cells were taken with 480/20 excitation filter, 525/40 emission filter (Chroma) using an Andor Clara 16-bit CCD camera. Images were processed using custom Matlab code [83]. Briefly, cells were segmented using the DIC channel. Fluorescence densities were computed by dividing total cell fluorescence by cell area. Independently, fluorescent foci were identifed in the GFP channel using local adaptive thresholding, and then mapped to cells by location. Maximal density was computed from the fluorescence of the brightest focus in the cell divided by the focus area. The ratio R between the maximal density and the density is used as a measure for aggregation.

Sample collection and alkaline lysis

Cell samples were collected at different times throughout theexperiments, as indicated. Cell density was determined as A_{600},identical numbers of cells were collected by centrifugation (13,000 rpm, 1 minute, 4°C), washed with 0.01 M NaNO₂, in phosphate-buffered saline (PBS), and frozen (~20°C), to allow simultaneous lysis. Importantly, aggregates and protein levels were not affected by freezing (data not shown). Cells were lyzed by incubation for30 minutes on ice in lysis buffer containing 0.2 M NaOH and5% (v/v) β-mercaptoethanol, pH was adjusted to 8.0 with 5N HCl and samples were boiled for 5 minutes.

Filter retardation and blotting assays

The protocol for measuring aggregation levels is based onmodified versions of previously described methods: aggregatesfiltration [96] and total protein dot blotting. Equal amounts ofboiled lysates were diluted 5-fold in either PBS or PBS supplemented with 2% (w/v) SDS (PBS/SDS). Samples in PBS wereapplied to PBS-soaked nitrocellulose membranes (0.2 μ; Protran Whatman) to absorb all proteins. Samples in PBS/SDS were filtered through nitrocellulose membrane soaked in PBS/SDS to retard only the aggregates. At least three independenttransformants were analyzed and triplicate samples from each(total of 9 samples) were tested for absorbance and retardationassays. The data are presented as mean ± SE. All samples wereapplied to a 96-well dot blotter (Biorad). Membranes were blockedwith 10% skim milk in PBS and polyQ proteins were detected byimmunoblotting.

Immunoblotting

The polyQ proteins were detected by a mouse anti-FLAGantibody (clone M2, Sigma). The secondary antibody used wasIRDye 800CW-conjugated goat anti-mouse (LI-COR Bioscienc-es). Alternatively, rabbit anti-GFP (ab290, Abcam) was used as aprimary antibody followed by goat anti-rabbit IgG DyLight 680-labeled (072-06-15-06, KPL). Secondary antibodies were visualized and quantified by the Odyssey Infrared Imaging System (LI-COR Biosciences).

Supporting Information

Figure S1 Quantitative microscopic assay reveals that25Q never aggregates. Wild-type cells (W303-1b) expressing25Q were grown under galactose induction for up to 42 hrs. At each time point hundreds of cells were imaged by fluorescence microscopy. Images were analyzed for the presence of aggregates in individual cells as presented by the ratio between the maximal density (y axis) and the density (x axis).

(TIF)

Figure S2 Quantitative microscopic assay reveals thataggregation of 47Q increases upon aging. Wild-type cells(W303-1b) expressing 47Q were grown under galactose induction for up to 42 hrs. At each time point hundreds of cells were imaged by fluorescence microscopy. Images were analyzed for the presence of aggregates in individual cells as presented by the ratio between the maximal density (y axis) and the density (x axis).

(TIF)

Figure S3 Quantitative microscopic assay reveals that103Q is always aggregated. Wild-type cells (W303-1b)expressing 103Q were grown under galactose induction for up to 42 hrs. At each time point hundreds of cells were imaged by fluorescence microscopy. Images were analyzed for the presence of aggregates in individual cells as presented by the ratio between the maximal density (y axis) and the density (x axis).

(TIF)

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Author Contributions

Conceived and designed the experiments: AC LR IN SB. Performed the experiments: AC LR. Analyzed the data: AC LR IN SB. Contributed reagents/materials/analysis tools: IN. Wrote the paper: AC IN SB.

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