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N-terminal truncations of human bHLH transcription factor Twist1 leads to the formation of aggresomes

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Abstract

In the cell, misfolded proteins are processed by molecular chaperone-mediated refolding or through ubiquitin-mediated proteasome system. Dysregulation of these mechanisms facilitates the aggregation of misfolded proteins and forms aggresomes in the juxta nuclear position of the cell which are removed by lysosome-mediated autophagy pathway in the subsequent cell division. Accumulation of misfolded proteins in the cell is hallmark of several neurological disorders and other diseases including cancer. However, the exact mechanism of aggresome formation and clearance is not thoroughly understood. Reports have shown that several proteins including p300, p53, TAU, α -synuclein, SOD, etc. contain intrinsically disordered region (IDR) which has the tendency to form aggresome. To study the nature of aggresome formation and stability of the aggresome, we have chosen Twist1 as a model protein since it has IDR regions. Twist1 is a bHLH transcription factor which plays a major role in epithelial mesenchymal transition (EMT) and shown to interact with HAT domain of p300 and p53. In the present study, we generated several deletion mutants of human Twist1 with different fluorescent tags and delineated the regions responsible for aggresome formation. The Twist1 protein contains two NLS motifs at the N-terminal region. We showed that the deletions of regions spanning the amino acids 30–46 (Twist1 30–46) which lacks the first NLS motif form larger and intense aggregates while the deletion of residues from 47 to 100 (Twist1 47–100) which lacks the second NLS motif generates smaller and less intense aggregates in the juxta nuclear position. This suggests that both the NLS motifs are needed for the proper nuclear localization of Twist1. The aggresome formation of the Twist1 deletion mutants was confirmed by counterstaining with known aggresome markers: Vimentin, HDAC6, and gamma tubulin and further validated by MG-132 treatment. In addition, it was found that the aggresomes generated by the Twist1 30–46 construct are more stable than the aggresome produced by the Twist1 47–100 construct as well as the wild-type Twist1 protein. Taken together, our data provide an important understanding on the role of IDR regions on the formation and stability of aggresomes.

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Keywords

Twist1; Nuclear localization Signal (NLS); Intrinsically disordered region (IDR); Misfolded proteins; Juxta nuclear position; Aggresome; Aggresome stability; Neurological disorder

Introduction

Proteins are the multifaceted biomolecules in the cell which serve many vital functions at multiple levels of almost all biological processes. All the synthesized polypeptides are not converted into functional proteins. After synthesis, protein folds in three-dimensional conformations based on their amino acids sequences and forms a proper structure for its localization and functions [1]. The steady-state levels of the proteins are maintained through ubiquitin proteasome system (UPS) and lysosomal-mediated degradation [2]. The failure of the proper folding under certain circumstances leads to the generation of the non-native or misfolded protein which in turn affects functions and localization of the protein [3]. Cells deploy different mechanisms to counteract the misfolded proteins. Most of the healthy cells refold the misfolded protein to its native conformation by up-regulating chaperones. If the refolding is not possible, then it degrades the misfolded proteins by UPS, which degrades misfolded proteins and smaller aggregates [4].

However, in the cell, when UPS turns dysfunctional and/or overwhelmed by cytosolic protein aggregates, another system called the aggresome comes into the action. The aggresome formation is a sequential process involving several other protein partners. Initially, the misfolded proteins are tagged with ubiquitin which in turn interacts with HDAC6 followed by Dynein-Dynactin motor complex. Subsequently, this misfolded protein complex moves to the centrosome (juxta nuclear position) through microtubule filament and forms a membrane less organelle/region called aggresome. This process is accompanied by the redistribution of intermediate filament protein vimentin to form a cage around the aggresome and these aggresomes are finally disposed/eliminated by lysosome-mediated autophagy [5–7]. Evidence also suggests that induction of autophagy leads to removal of the cells which harbor aggresomes while the inhibition of autophagy leads to the formation of stronger aggresome [8].

Aggregation of many misfolded proteins results in lethal disorders including neurodegenerative diseases, Alzheimer's, Parkinson's, Huntington, dementia with Lewy bodies, Amyotrophic Lateral Sclerosis, Down's syndrome, etc., indicating the importance of the aggresome clearance pathways. Often, the aggresome forming proteins are intrinsically disordered proteins (IDP) which contain intrinsically disordered regions [IDR]. The IDR have no ordered secondary or tertiary structures being very flexible to change the conformation of the native protein [9, 10]. Several important proteins like p300 (transcriptional coactivator), p53 (tumor suppressor), p21 (cell cycle inhibitor), Ataxin-3 (deubiquitinase), α -synuclein (presynaptic neuronal protein), SOD1 (superoxide dismutase), Tau (microtubule-associated protein), E6AP (ubiquitin-protein ligase), CFTR (membrane protein and chloride channel) have been reported to contain IDR and form aggresomes [11–18].

Twist1, a basic Helix Loop Helix (bHLH) transcription factor is one such IDR containing protein which forms homodimer and/or heterodimer with its own and other bHLH proteins respectively to perform its functions [19]. Mutations in Twist1 cause craniofacial development disorder called Saetho Chotzon Syndrome (SCS) [20] and Robinow-Sorauf Syndrome [21]. Twist1 has a large IDR in the N-terminus region (residues 3–102) [22] which comprises a glycine-rich region (residues 47–98) and two nuclear localization signals: NLS1 and NLS2 at the positions of 37–40 and 73–77, respectively. C-terminus of Twist1 comprises DNA binding bHLH domain and Twist box which is known to interact with p53, RUNX2, etc. [23–26]. Limited studies have been conducted for the N-terminal IDR region of Twist1 compared to its C-terminal region. Reports have shown that Twist1 physically interacts with other IDR proteins, particularly, p300 and p53 [25, 27]. Twist1 is also shown to upregulate the expression of aggresome marker protein: vimentin [28]. These information prompted us to investigate N-terminal IDR region of Twist1 for the formation of aggresomes.

In the present study, we examined the sub-cellular localization of various N-terminal deletions of Twist1 and showed for the first time that deletion of IDR in the N terminal regions of Twist1 leads to the formation of aggresomes and it is not a cell line specific. The aggresome formation by the N-terminal truncations of Twist1 was confirmed by co-localization and immunostaining using aggresome-specific markers. Overall, our data demonstrate that Twist1 IDR region plays a major role in proper folding and its deletion leads to aggresome formation.

Materials and methods

Cell culture

HEK293, human embryonic kidney cells were obtained from National Centre for Cell Sciences, Pune, INDIA and cultured in Dulbecco's modified Eagles's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), (HiMedia, Cat # RM1112) and 200 mM L-glutamine-penicillin-streptomycin (HiMedia, Cat # A007) at 37°C incubator with 5% CO₂ atmosphere. T98G, human glioblastoma cell line was obtained from Professor Kumaravel Somasundaram, Indian Institute of Science (IISc), Bangalore, INDIA as a gift and grown as mentioned above. Transfection reagent Turbofect was purchased from Thermofisher scientific (Cat # R0531) and used according to manufacturer's instruction. Cycloheximide (Cat # C104450) and MG132 (Cat # M7449) were purchased from Sigma and used at 20 µg/ml and 5 µM concentrations, respectively.

Generation of WT-Twist1 and deletion constructs

Total RNA was prepared from HEK293 cell line by Trizol reagent (Invitrogen, Cat # 15596-026) and cDNA was synthesized from 2 micrograms of total RNA using first strand cDNA synthesis kit (Invitrogen, Cat # 18080-051). For the generation of WT-Twist1 construct, HEK 293 cDNAs were PCR amplified using Twist1 wild-type (WT)-specific primers and cloned in frame in pDsRED-C1 vector at XhoI-BamHI sites. The Twist1 deletion 1–29 (1–29) was subcloned in pDsRED-C1 from WT-Twist1-RED-C1 clone using specific primers by standard cloning method. Clones of Tw1 30–46 and Tw1 47–100 were

generated in pDsRED-C1 by site-directed mutagenesis (SDM) using WT-Twist1-RED-C1 as a template. Briefly, WT-Twist1-RED-C1 template was amplified using Pfu Ultra high-fidelity DNA polymerase with specific primers, digested with Dpn1, transformed in XL1Blue competent cells and screened for positive colonies by colony PCR. Clones were sequenced using vector and gene-specific primers and confirmed. Twist1 deletion constructs in pEGFP-C2 and pDsRED-N1 were subcloned from pDsRed-C1 clones by standard cloning. Full length HDAC6 and γ -tubulin were PCR amplified from HEK293 cDNA using gene-specific primers and cloned in pEGFP-C2. Plasmids; pDsRED-C1, pDsRED-N1 and pEGFP-C2 were received from Professor. Narkunaraaja Shanmugam, Bharathidasan University, INDIA as gift. Primers employed for cloning is tabulated in Supplementary (S1). CUL4A-GFP construct was described in our earlier report [29].

Transfection and immunofluorescence

The cells (HEK293 or T98G) seeded in 35-mm dishes containing autoclaved glass coverslips were transfected with appropriate Twist1 constructs by Turbofect transfection reagent following the manufacturer's protocol. Briefly, 0.5×10^6 cells were seeded and cultured for 24 h. For transfection, 4 μ g of plasmids (for co-transfection, 2 + 2 μ g of two different plasmids) was mixed with turbofect reagent in DMEM and incubated for 20 min at RT. After incubation, the transfection complex was added to the cells and cultured. At desired time points, the cells in the coverslips were fixed in 3.7% formaldehyde, washed with PBS, and stained with DAPI to visualize nuclei. Finally, the coverslips were mounted on the glass slide using mounting media Mowiol (Sigma, Cat # 81381) containing the anti-fade reagent DABCO [1, 4-Diazabicyclo [2.2.2] octane] (Sigma, Cat # D27802). The images were captured at different magnifications ($\times 20$ and $\times 40$) using Nikon TiS or the confocal microscopy Zeiss LSM 700.

Immunostaining

Cells grown on coverslips were transfected with Twist1-GFP deletion construct and immunostained at desired time points using specific antibodies as mentioned below. Transfected cells were fixed in 3.7% formaldehyde (15 min), washed with PBS, permeabilized with 0.2% Triton X-100 in PBS (5 min) and then blocked with 3% BSA in PBS for at least 30 min at RT. The cells were washed once and specific primary antibodies [anti-Vimentin (Santa Cruz, Cat # SC-6260), anti-HDAC6 (Santa Cruz, Cat # SC-28386), and anti- γ tubulin (Sigma, Cat # T6557)] were added in a freshly prepared 0.5% BSA in PBS (1:500) and incubated for 2 h at RT. Following seven PBS washes, the secondary antibodies conjugated to Alexa Fluor 555 (Invitrogen, Cat # A-21422) were added (1:1000 in PBS) to the cells and incubation continued for 1 h at RT in dark. Finally, the cells were washed ten times with PBS and stained with DAPI. Finally, the coverslips were mounted on glass slide using mounting media Mowiol-DABCO and images were captured at different magnifications using Nikon TiS. Fluorescence pictures were merged using Image J software.

Twist1 deletion overexpression, cycloheximide half life chase and immunoblotting

For Twist1 overexpression, HEK-293 cells were transfected with Twist1-WT, 30–46, and 47–100 constructs using turbofect reagent and cultured for 36 h. The transfected cells were washed with ice-cold PBS, harvested and resuspended in RIPA lysis buffer (50 mM Tris HCl

pH 8.0, 150 mM NaCl, 1% IGEPAL CA630, 0.5% Sodium deoxycholate, 0.1% SDS, 5 mM EDTA) with protease inhibitor (Roche Cat # 04 693 159 001). After 20 min of incubation on ice, centrifuged at 12000 rpm for 15 min and whole cell extracts in the supernatant were collected in a fresh tube. Equal concentration of whole cell extracts was resolved on 12% SDS-PAGE gel, transferred on nitrocellulose membrane, and blocked in 5% skim milk in Tris buffered saline (TBS) containing 0.1% Tween (TBST) for 1 h at RT. The membrane was washed three times in TBST and incubated with primary antibody against Twist1 (Santa Cruz, Cat # SC-81417), β -actin (Santa Cruz, Cat # SC-47778) for 1–2 h at RT. Following three washes (TBST), the secondary antibody goat Anti-Mouse IgG (H+L)-HRP Conjugate (Bio-Rad, Cat # 170-6516) was added, incubated for 45 min and washed three times in TBST. The protein bands were detected using ECL detection system (Roche, Cat # 11500708001). For cycloheximide treatment, Twist1 WT, 30–46, 47–100 overexpressed cells at 36 h were treated with 20 μ g/ml of cycloheximide, harvested at different time points as indicated and analyzed Twist1 protein by immuno blotting using Twist1-specific antibodies. β -actin was included as an internal loading control.

Results

Effect of N-terminal deletions on the sub-cellular localization of Twist1

To study the functional importance of the N-terminal IDR regions of Twist1, we constructed several deletion mutants on N-terminal regions of Twist1 namely, 1–29, 30–46, and 47–100 in pDsRED-C1 vector. The schematic representation of Twist1 deletion constructs used in this study is depicted in Fig. 1a. These Twist1 deletions were overexpressed in fusion with C-terminal of red fluorescent protein (DsRed-monomer) by transient transfection in HEK293 cells and analyzed their sub-cellular localization under fluorescence microscope. As expected, the wild-type Twist1 (WT-Twist1) which contains both NLS showed homogenous distribution in the nucleus (Fig. 1b(i)). Deletion of first 29 amino acids of Twist1 showed nuclear localization with intense spots in the nucleus, and the pattern was different from the WT-Twist1 nuclear localization (Fig. 1b(ii)).

Interestingly, the deletion construct of Twist1 which lacks the NLS1 (residues 30–46) (30–46) displayed strong aggresome-like structure in juxta nuclear position (Fig. 1b(iii)) while the deletion construct of Twist1 which lacks the NLS2 (residues 47–100) (47–100) also exhibited aggresome-like structure in juxta nuclear position but the size and intensity of the aggregates are less in comparison to the 30–46 construct (Fig. 1b(iv)). The large aggregate of Twist1 30–46 is also visible even in the phase contrast images of confocal laser scanning microscopy (Fig. 1c). Despite the presence of one intact NLS in these 30–46 and 47–100 mutants failed to enter nucleus and showed juxta nuclear localization pattern suggesting that both the NLS sequences are required for nuclear import of Twist1.

N-terminal deletions of Twist1 form aggresomes

The juxta nuclear localization pattern is the peculiar property of the accumulated misfolded protein in the form of aggresomes and hence we investigated the aggresome formation by Twist1 30–46 overexpression by colocalization and immunostaining with aggresome-specific marker proteins. MG132, a common proteasomal inhibitor used in cell culture to

imitate the cellular aggregates which is known to form large aggresomes in the juxta nuclear area was included as a positive control in our experimental design [30, 31]. Our fluorescence images revealed that the localization pattern observed in Twist1 30–46 overexpressed cells is similar to the MG132-induced aggresomes (Fig. 1b(iii) and Supplementary Fig. S2). In addition, we also noted the reported localization of HDAC6 (aggresome marker) [6], γ -tubulin (centrosome marker), CUL4A (Centrosome associated E3 ligase) [29] in the juxta nuclear position when cells were treated with MG132 (Supplementary Fig. S2).

Our co-transfection studies from Twist1 30–46-RED with aggresome markers proteins, HDAC6-GFP and γ -tubulin-GFP, clearly showed the perfect co-localization suggesting that the observed aggregates of 30–46 truncated proteins of Twist1 are indeed a part of aggresome (Fig. 2a). The colocalization of 30–46 truncated proteins with aggresome marker proteins was further confirmed by immunostaining using aggresome marker protein-specific antibodies (anti-HDAC6, anti- γ tubulin, and anti-vimentin) (Fig. 2b). We have previously shown that the CUL4A interacts with γ tubulin and localized in the centrosome [29], and hence, we have also investigated the CUL4A localization in the aggresomes. It was found that CUL4A also co-localizes with aggresome formed by Twist1 30–46 (Fig. 2c).

It is well reported that type and location of fluorescent tag (N-terminus or C-terminus) often affect the localization of overexpressed fusion proteins [32, 33]. Since we noted the juxta nuclear aggregates with the Twist1 30–46 construct, we have studied the effect of different fluorescent tag and its location on the sub-cellular localization of Twist1 30–46 proteins. To this end, Tw1 30–46 in pDsRED-C1 (Tw1 30–46 RED-C1) was subcloned in pDsRED-N1 (Tw1 30–46 RED-N1) and pEGFP-C2 (Tw1 30–46 GFP-C2) and studied their localization pattern in HEK293 cells. It was found that the localization and formation of aggregates by Twist1 30–46 proteins remain unchanged suggesting that the observed pattern was not due to the fusion protein and/or position of the fluorescent tag (Fig. 2d). We also investigated the formation of aggresome by Twist1 30–46 in the glioblastoma cell line T98G. The Twist1 30–46 proteins formed a larger aggregates at juxta nuclear position similar to the pattern observed in 293 cells suggesting that the formation of aggresomes by Twist1 30–46 is not cell specific (Fig. 2e).

Stability of Twist1 30–46 and Twist1 47–100 induced aggregates

Our transient transfection followed by microscopic observation revealed that time taken for formation and appearance of the aggregates differs significantly among Twist1 30–46 and 47–100 induced aggregates. We have analyzed the formation of aggregates 30–46 and 47–100 constructs at 24, 36, and 48 h of post-transfection and it was found that 30–46 construct formed aggregates at earlier time points than aggregates formed by 47–100 construct (Fig. 3a). In addition, the number of aggregate forming cells in 30–46 overexpression was higher (nearly four folds) than Twist1 47–100 overexpression (Fig. 3b).

Since, we observed the noticeable difference of aggresome sizes and the intensity of fluorescence among Twist1 deletions, next, we investigated the stability of these Twist1 30–46- and Twist1 47–100-induced aggregates by using cycloheximide drug treatment. The levels of overexpressed proteins from Twist1 deletion constructs were analyzed by immunoblotting using Twist1-specific antibodies and found that the quantity of the proteins

is similar (Fig. 3c). Our cycloheximide half-life chase experiment clearly showed that the Twist1 30–46-induced aggregates which lack NLS1 are more stable compared to Twist1 47–100 which lacks NLS2 and also WT-Twist1 protein (Fig. 3d). β -actin was included as an internal loading control. These data clearly indicate that the nature of amino acid deletion in the N-terminal of Twist1 including NLS motif determines the folding of Twist1, nuclear entry, and its stability.

Since the Twist1 30–46 proteins formed larger aggregates and displayed enhanced stability compared to WT-Twist1 and Twist1 47–100, the amino acid region of 30–46 plays a vital role in determining the folding and localization of Twist1 protein.

Discussion

Aggresome represents a protective cellular response to the buildup of aggregating abnormal polypeptides when chaperones and the UPS fail to handle abnormal species [3, 4]. Although the process of aggresome formation and clearance has been elucidated to some extent from several studies, the knowledge on aggresome formation by different proteins particularly transcription factor(s) is not thoroughly understood. As mentioned earlier, special machinery has evolved to transport such aggregates to the centrosome in a microtubule-dependent manner which forms a membrane less organelle called an aggresome. These aggresome in the cell is ultimately cleared by lysosome-mediated autophagy pathway [5–7]. Aggresome is also cleared or diluted through replicative rejuvenation process upon subsequent cell divisions where one of the daughter cells becomes free of aggresome but the molecular mechanisms behind this phenomenon are also not completely known [34]. It is reported that there is strong correlation between aggresome formation and cell survival [35]. Multiple lines of evidence suggest that the autophagic machinery gets recruited to aggresome, thus leading to its disassembly [36, 37].

Here, we focused on N-terminal region (1–100 amino acids) of human Twist1, because it contains IDR regions and also shown to interact with other IDR containing proteins including p300 which is strongly associated in almost all the aggregates which form aggresome. It is well known that the deletion of NLS should block the nuclear entry and localize in the cytoplasmic regions as diffused. Previous report showed that the point mutations made in Twist1 NLS regions result in diffused cytoplasmic localization of the constructs [25]. Here, we have shown for the first time that the destruction/deletion of N-terminal regions including at least one NLS of the transcription factor Twist1 leads to an aggregate due to its misfolded proteins and forms aggresome in the juxta nuclear position. The aggresome localization pattern which we observed in Twist1 N-terminal deletions was similar to MG132-induced aggresome. We also found that the Twist1-induced aggresome formation is due to the fusion protein and or its position of tag used. Twist1 deletion mutants with at least one NLS intact failed to enter the nucleus which clearly portrayed that both NLS motifs along with the flanking amino acid regions of Twist1 protein are needed for its nuclear entry. In addition, we observed noticeable differences among aggresomes formed by different Twist1 deletions. For example, in Twist1 30–46, the deletion of 17 aa including NLS1 forms a large size aggresome, whereas 53 aa deletion including NLS2 in Twist1 47–100 forms smaller aggresome.

Based on our observation, it is very apparent that the N-terminal sequences of Twist1 along with NLS motifs (especially amino acid region 30–46) play a crucial role on proper folding of Twist1 protein. Observation of stability using cycloheximide pulse chase shows that the Twist1 30–46-induced aggregate is more stable than Twist1 47–100 and WT-Twist1 which confirms the importance of the particular region on its proper folding and stability. The reason behind the varied stability and the time taken for the occurrence of aggregates between different deletions of Twist1 could be due to several parameters which include the size of the deletion, nature and folding of the amino acids in the deleted region, functional status of the proteasome degradation machinery, etc. Nevertheless, a detailed mutagenesis study is required to prove the exact role (s) of Twist1 NLS and its flanking sequences on aggresome formation.

The aggresome observed in two different cell lines (HEK293 and T98G) suggesting that the aggresome formation, at least Twist-induced aggresome, is not a cell line specific. Reports have suggested that HDAC6 binds to polyubiquitinated misfolded proteins by its zinc finger and transport them to the aggresome [38] and since our immunostaining study showed the colocalization of HDAC6 with the Twist1 aggregates, we commemorate these Twist1 deletion-induced aggregates are polyubiquitinated which might undergo lysosome mediated autophagy pathway for its clearance.

Earlier, we showed that CULLIN4A, an E3 ligase protein, physically interacts with γ -tubulin and also co-localized in the centrosome region [29]. Since CUL4A plays a major role in degradation of target proteins through polyubiquitination followed by 26S proteasome degradation machinery, here, we observed the localization of CUL4A in Twist1 30–46 overexpressed cells and found the co-localization of CUL4A protein with Twist1 aggregates (Fig. 2c). Although, we observed the co-localization of CUL4A and γ -tubulin with Twist1 deletion protein-induced aggregates, their exact role(s) on aggresome mechanism are yet to be investigated. It also enlightens the idea that the CUL4A protein and possibly, other unidentified RING E3 ligase protein(s) might be associated in the aggresome complex which get triggered (become active) when cells enter into subsequent cell division where aggresomes are cleared.

Together, our data demonstrated for the first time that the deletion of amino acids in N-terminal regions encompassing NLS motifs of Twist1 bHLH transcription factor results in misfolding and aggresome formation. This information will provide extra informations on aggresomes and will open up several follow-up studies particularly in the area of neurogenerative medicine ranging from aggresome formation to its clearance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Sub-cellular localization of WT and N terminal deletions of Twist1. **a** Schematic representation of N-terminal deletions constructs of Twist1 used in this study. Aggresome details (+ or -) given in the schematic are based on our observation. **b** HEK293 cells were transfected with Tw1-WT (i), Tw1- 1-29 (ii), Tw1- 30-46 (iii), and Tw1- 47-100 (iv) and analyzed the sub-cellular localization pattern at 36 h under fluorescence microscope **c** Confocal image of HEK293 cells overexpressing Twist1 30-46 clearly shows the juxta nuclear aggregate localization

Fig. 2.

N-terminal deletions of Twist1 form aggresomes. **a** HEK293 cells were co-transfected with Twist1 30–46 RED-C1 and aggresome markers; HDAC6-GFP (*Top*) or γ -tubulin-GFP (*Bottom*) and analyzed the co-localization pattern under fluorescence microscope. **b** Twist1 30–46 overexpressed HEK293 cells were immunostained (36 h) with aggresome markers protein-specific antibodies followed by secondary antibodies conjugated with alexafluor 555 and analyzed the co-localization as mentioned above. **c** Co-transfection of 30–46 RED-C1 with CUL4A-GFP showing co-localization of CUL4A with aggresome. **d** Twist1 30–46

cloned in pDsRED-C1 (Tw1 30–46 RED-C1), pDsRED-N1 (Tw1 30–46 RED-N1), and pEGFP-C2 (Tw1 30–46 GFP-C1) showing similar juxta nuclear localization patterns (*Top left, Top right and Bottom panels* respectively) **e** 30–46 RED-C1 (*Bottom*) transfected T98G showing very strong juxta nuclear localization (*Top*)

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Fig. 3.

Stability of N-terminal Twist1-induced aggregates. **a** Images of juxta nuclear aggregates of Twist1 N-terminal deletions and the 24-, 36- and 48-h post-transfection showing the early and delayed occurrence of aggregates in Δ 30-46 and Δ 47-100 constructs respectively under constant exposure time; 1 s. **b** Average number of cells (HEK293) harboring fluorescent aggregate from Δ 30-46 to Δ 47-100 overexpressed cells (36 h). Values are from three different microscopic fields. **c** The whole protein extracts prepared from the cells overexpressing WT and Twist1 deletions were immunoblotted using anti-Twist1 antibodies

d WT and 30–46 and 47–100 constructs overexpressed HEK293 cells (36 h) were treated with cycloheximide and at the indicated time points, the protein levels were analyzed by immunoblotting using anti-Twist1 antibodies. β -actin was shown as an internal loading control

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