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IDENTIFICATION AND CHARACTERIZATION OF ACTIN-BINDING MOTIFS ON THE
ANDROGEN RECEPTOR AND HUNTINGTIN: IMPLICATIONS FOR
POLYGLUTAMINE DISEASE

by

Suzanne Irene Angeli

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOMEDICAL SCIENCES

in the

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of the

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by

Suzanne Irene Angeli

For my parents:

Roberto and Amanda Angeli

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IDENTIFICATION AND CHARACTERIZATION OF ACTIN-BINDING MOTIFS ON
THE ANDROGEN RECEPTOR AND HUNTINGTIN: IMPLICATIONS FOR
POLYGLUTAMINE DISEASE

Suzanne Irene Angeli

Polyglutamine diseases are a devastating class of inherited, neurodegenerative disorders, with protein misfolding as a hallmark. Spinobulbar muscular atrophy (SBMA) and Huntington's disease (HD) are two distinct polyglutamine diseases caused by an expanded polyglutamine tract in the androgen receptor (AR) or huntingtin (Htt) protein, respectively. An expanded polyglutamine tract is thought to induce protein misfolding, aggregation, and toxicity. N-terminal fragments of AR or Htt, which contain the polyglutamine stretch, are believed to be the toxic species in these diseases. It has previously been reported that actin-regulatory pathways can modulate intracellular aggregation of N-terminal fragments of AR and Htt. Sequences that flank the polyglutamine tract of AR and Htt might influence protein aggregation and toxicity through direct protein-protein interactions, but this has not been studied in detail.

In this study, we have evaluated an N-terminal 127 amino acid fragment of AR (ARN127) or Htt exon 1. We find that the first 50 amino acids of ARN127 (ARN50) and the first 14 amino acids of Htt exon 1 (HttN14) directly mediate binding to filamentous actin (F-actin) *in vitro*. ARN127 and Htt exon 1 bind to F-actin in a saturable manner, with affinities that range between 1-2 μ M. ARN50 and HttN14 are both enriched in Triton-insoluble fractions of cell lysates, indicating tight interactions with membranes and/or large cytoskeletal proteins. ARN50 and HttN14 promote the formation of polyglutamine inclusions and deletion of these actin-binding regions renders the

polyglutamine-expanded forms of ARN127 and Htt exon 1 to become more soluble. ARN50 is required for sensitivity to actin-regulatory compounds, regulates the type, number, and distribution of polyglutamine inclusions, and promotes SDS-insolubility of aggregates. Though HttN14 does not appear to drastically regulate the types of inclusions formed, it too promotes a biochemically distinct SDS-insoluble form of aggregate. The F-actin binding regions on ARN127 and Htt exon 1 thus appear to alter the aggregation frequency and type of polyglutamine-induced aggregation. These findings highlight the importance of protein interactions via flanking sequences in determining the propensity of unstable proteins to misfold.

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Chapter 1

Introduction

Introduction

Polyglutamine diseases are a devastating class of inherited, progressive, neurodegenerative diseases (for a comprehensive review, please see Zoghbi and Orr, 2000). There are currently nine known polyglutamine diseases (Table 1) (Zoghbi and Orr, 2000). Polyglutamine diseases are caused by an aberrantly long stretch of trinucleotide repeats (CAG_n) encoded in the DNA of a specific gene (Zoghbi and Orr, 2000). Transcription and translation of this gene lead to expression of a protein that contains a consecutive stretch of glutamines (polyglutamine) (Zoghbi and Orr, 2000). Most polyglutamine diseases are autosomal dominant, with the exception of spinobulbar muscular atrophy, which is X-linked (La Spada et al., 1991; Zoghbi and Orr, 2000). All polyglutamine diseases involve a unique protein with no known relationship to other polyglutamine proteins (Table 1). Despite this, all polyglutamine diseases share many characteristics, which will be briefly discussed below. *i.* The inheritance of a polyglutamine disease is typically characterized by *anticipation*, in which subsequent generations experience earlier age of onset and more severe disease progression (Zoghbi and Orr, 2000). *ii.* All polyglutamine diseases exhibit a polyglutamine repeat *threshold* for disease onset (Table 1). Surpassing a critical number of glutamines in a protein, usually around 40, although it depends on the specific polyglutamine protein, is sufficient to cause disease (Zoghbi and Orr, 2000). *iii.* The age of onset for polyglutamine diseases is inversely correlated with the length of polyglutamine tract (1993; Andrew et al., 1993; Duyao et al., 1993; La Spada et al., 1991; Snell et al., 1993). *iv.* All polyglutamine diseases display selective neurodegeneration, despite ubiquitous expression of some polyglutamine proteins (Table 1) (Zoghbi and Orr, 2000). *v.* All polyglutamine diseases

are characterized by the presence of polyglutamine inclusions, very large aggregates that form in neurons and tissues of patients (Zoghbi and Orr, 2000). Thus, it is believed that protein misfolding due to the enlarged polyglutamine tract underlies the pathogenesis of all these diseases. Since different polyglutamine diseases can exhibit different regions of neural degeneration, it has been suggested that cell-type vulnerabilities, potentially due to specific protein-protein interactions, may contribute to the distinct neuropathological features of each disease (Subramaniam et al., 2009). Thus, discovering cell-type specific interactions for each polyglutamine disease will be important in finding therapeutic leads.

Spinobulbar Muscular Atrophy

Androgen Receptor.

Spinobulbar muscular atrophy (SBMA), also known as Kennedy's disease (Kennedy et al., 1968), was the first polyglutamine disease to be genetically identified and heralded a new era of polyglutamine disease research (La Spada et al., 1991). It is caused by an expanded polyglutamine stretch on the androgen receptor (AR), located on chromosome Xq11-q12 (La Spada et al., 1991). SBMA is one of the few polyglutamine diseases in which the culprit protein has a clear, well-defined function. AR is part of the nuclear receptor superfamily that includes the estrogen receptor (ER), glucocorticoid receptor (GR), and mineralcorticoid receptor (MR) (Beato and Klug, 2000; Quigley et al., 1995). Nuclear receptors are composed of three regions: an N-terminal transactivation domain (TAD), a central DNA binding domain (DBD), and a C-terminal ligand-binding domain (LBD) (Beato and Klug, 2000; Quigley et al., 1995). In its quiescent state, AR is cytoplasmic and complexes with heat shock proteins. Upon binding to androgens

(testosterone or dihydrotestosterone), AR undergoes a conformational change, translocates into the nucleus, and homodimerizes. In the nucleus, the AR dimer binds hormone response elements and initiates transcription of target genes (Beato and Klug, 2000; He and Wilson, 2002; Quigley et al., 1995). During this process, AR recruits coactivators that can facilitate nuclear translocation and AR-mediated transcription of target genes (Heinlein and Chang, 2002; Lee and Chang, 2003). To date, numerous coregulators have been described for AR (Heinlein and Chang, 2002). Further, there is growing evidence that cytoskeletal proteins may represent a new faction of steroid receptor coregulators, which will be discussed more in detail in Chapter 2.

SBMA Clinicopathology.

SBMA is characterized by muscle weakness and fasciculations, difficulty swallowing, and gynecomastia (Harding et al., 1982; Kennedy et al., 1968). Symptoms usually begin to manifest 30-50 years of age (Harding et al., 1982; Kennedy et al., 1968). The predominant areas of neural degeneration are primary sensory and lower motor neurons (Sobue et al., 1989). SBMA occurs only in men, as the toxic effects of polyglutamine-expanded AR are dependent on testosterone activation (Katsuno et al., 2002; Li et al., 2007). Women homozygous for the mutation exhibit only very mild symptoms, and female mice models of SBMA exhibit pathological features only when administered testosterone (Katsuno et al., 2002; Schmidt et al., 2002). Complete loss of function of AR leads to a completely different disorder known as androgen insensitivity syndrome (AIS), in which males appear as phenotypic females (Quigley et al., 1995).

Thus, like all polyglutamine diseases, a toxic gain of function is thought to underlie pathogenesis.

Huntington's Disease

Huntingtin.

In 1993, the Huntington's Disease Collaborative Research Group identified novel gene IT15 on chromosome 4p16.3 as the cause of HD (1993). IT15 encodes a very large, ~350 kDa protein, which was termed huntingtin (Htt). Huntingtin is a cytoplasmic protein widely expressed in many tissues, including the brain (DiFiglia et al., 1995). The exact function of Htt remains unclear, though it has been implicated in transcriptional regulation, anti-apoptotic pathways, and vesicular recycling and transport (Caviston and Holzbaur, 2009; Shao and Diamond, 2007). Knockout of the Htt gene in mice results in embryonic lethality, underscoring its developmental importance (Nasir et al., 1995; Zeitlin et al., 1995). It contains no known homology to other proteins other than HEAT domains (Huntingtin, Elongation factor 3, protein phosphatase 2A, TOR1), which are thought to mediate protein-protein interactions (Andrade and Bork, 1995).

HD Clinicopathology.

Huntington's disease (HD) is the most common and widely studied of all polyglutamine diseases. It was described by George Huntington in an 1872 article in which *chorea*, an involuntary, dance-like writhing exhibited by patients, was especially noted (Huntington, 1872). In addition to motor dysfunctions, it is characterized by changes in personality and other cognitive abnormalities (Walker, 2007). The primary

areas of neural degeneration are medium spiny neurons of the caudate and putamen, though other areas are also often affected (Vonsattel and DiFiglia, 1998; Vonsattel et al., 1985). It is inherited in an autosomal dominant fashion. Homozygotes for the mutation have been reported to show no increase in disease severity or progression (Wexler et al., 1987), underscoring the true dominant nature of the disease.

Pathogenesis of SBMA and HD

Like many polyglutamine diseases, SBMA and HD share some pathogenic characteristics. Aberrant proteolytic cleavage of full-length AR and Htt appears to generate toxic, N-terminal fragments (Goldberg et al., 1996; Li et al., 2007; Li et al., 1998; Wellington et al., 2000), which are sufficient to recapitulate neurodegenerative phenotypes *in vivo* (Abel et al., 2001; Davies et al., 1997). N-terminal fragments of expanded AR and Htt readily aggregate *in vitro* and in cell-culture models, thus making them useful in biochemical studies (Merry et al., 1998; Pollitt et al., 2003; Scherzinger et al., 1997). The rate of polyglutamine aggregation directly correlates with the length of the polyglutamine tract and protein concentration *in vitro* (Scherzinger et al., 1999). Studies have also implicated the biophysical properties of flanking amino acids as important regulators of polyglutamine aggregation *in vitro* (Bhattacharyya et al., 2006; Thakur et al., 2009). Flanking sequences can also potentially unmask polyglutamine toxicity intracellularly, either by changes in conformation and/or interactions with proteins (Duennwald et al., 2006a; Duennwald et al., 2006b). In addition, post-translational modifications on polyglutamine proteins can greatly influence aggregation and toxicity (Aiken et al., 2009; Steffan et al., 2004), as can signaling pathways that

likely act via protein-protein interactions (Diamond et al., 2000; Humbert et al., 2002; Pollitt et al., 2003). While molecular chaperones have been commonly shown to modulate polyglutamine aggregation (Behrends et al., 2006; Muchowski et al., 2000; Tam et al., 2006; Tam et al., 2009; Wacker et al., 2004), other protein-protein interactions not associated with general housekeeping have not been studied in great detail. Emerging evidence from a variety of studies of aggregation-prone proteins associated with neurodegenerative diseases suggests that there is considerable diversity among aggregates that can be formed *in vitro* (Frost et al., 2009; Muchowski et al., 2000; Nekooki-Machida et al., 2009) and, moreover, that some protein aggregates are likely to be more toxic than others (Nekooki-Machida et al., 2009; Petkova et al., 2005). Thus, protein interactions that alter aggregate conformation could play an important role in determining toxicity.

Actin as a Potential Modulator of Polyglutamine Aggregation

Previous work has implicated the actin cytoskeleton as a potential modulator of polyglutamine diseases. Actin is an essential cytoskeletal protein that can exist in two forms, monomeric or globular (G-actin) or filamentous (F-actin) (dos Remedios et al., 2003). Intracellular inclusions isolated from HEK293 cells expressing a polyglutamine-expanded N-terminal fragment of Htt revealed that many proteins are sequestered, including actin (Suhr et al., 2001). In yeast, cortical F-actin patches (thought to be sites of endocytic vesicle formation) also colocalized with Htt inclusions (Meriin et al., 2003). This sequestration could indicate a direct interaction with the polyglutamine fragment, or represent a non-specific recruitment of actin. Manipulation of the actin cytoskeleton has

been shown to modulate polyglutamine aggregation. In both yeast and mammalian cells, inhibition of actin polymerization by the actin-depolymerizing drug latrunculin A enhanced Htt aggregation (Meriin et al., 2007). In mammalian cells, siRNA or chemical inhibition of N-WASP, an upstream effector of actin-organization (Mullins, 2000), inhibited Htt aggregation, while Arp2, a protein involved in the formation of actin branches (Mullins, 2000), enhanced Htt aggregation (Meriin et al., 2007). Thus, while these data point to actin dynamics as a modulator of polyglutamine aggregation, it remains unclear how or if actin is directly involved.

We previously performed a screen to identify small molecule inhibitors of polyglutamine aggregation using a fluorescence resonance energy transfer (FRET) based cellular assay (Pollitt et al., 2003). N-terminal fragments of either AR (the first 127 amino acids of AR, ARN127) or Htt exon 1 tagged to either donor or acceptor FRET pairs (CFP and YFP) were used. We found that Y-27632, a rho-kinase (ROCK) and protein-kinase C-related protein kinase (PRK-2) inhibitor (Amano et al., 2000; Davies et al., 2000), decreased polyglutamine aggregation up to 20% for ARN127 and Htt exon 1 (Pollitt et al., 2003; Shao et al., 2008a). It was subsequently found that Y-27632 can also reduce ataxin-3 and atrophin-1 polyglutamine aggregation (Bauer et al., 2009). Y-27632 was shown to reduce toxicity in a *Drosophila* model of HD and improved motor performance in transgenic HD mice (Li et al., 2009; Pollitt et al., 2003). Rho-kinase and PRK-2 regulate many downstream effectors that reorganize the actin cytoskeleton (Amano et al., 2000; Vincent and Settleman, 1997). Thus, Y-27632 may mediate its anti-aggregation effects via regulation of the actin cytoskeleton. It has also been suggested

that Y-27632 may cause a general activation of degradation pathways, which contributes to its anti-aggregation effects (Bauer et al., 2009).

Further evidence that actin regulation is closely tied to polyglutamine aggregation came when we found that Y-27632 blocks ROCK-mediated phosphorylation of profilin, a canonical G-actin binding protein (Witke, 2004) that directly binds Htt, but not AR (Shao et al., 2008b). Profilin strongly inhibits aggregation of ARN127 and Htt exon 1 in cells (Shao et al., 2008b) and decreases polyglutamine-mediated toxicity in *Drosophila* (Burnett et al., 2008). The anti-aggregation effects of profilin depend on both its polyproline binding activity (likely required to bind Htt), and its ability to bind G-actin (required to suppress both Htt and AR aggregation) (Shao et al., 2008b). Given that profilin's actin-binding activity is required to reduce aggregation, this strongly implicates actin and actin dynamics as a key regulator of polyglutamine aggregation.

In PC12 cell-lines, expanded Htt has been reported to enhance profilin degradation, and decreased amounts of profilin have been reported in HD patients (Burnett et al., 2008). Profilin loss due to expanded Htt also appears to perturb F:G-actin ratios in cells, which are restored by profilin (Burnett et al., 2008). Thus, profilin dysfunction via protein interactions and/or regulation of the actin cytoskeleton may contribute to the underlying pathogenesis of polyglutamine disease, though this remains to be explored.

Concluding Remarks

Given the potential importance of specific protein-protein interactions mediating cell-type susceptibility to neurodegeneration, and the unexplored and unknown

importance of actin in polyglutamine aggregation, we have investigated the relationship between actin and the polyglutamine proteins, AR and Htt.

Table 1

| Disease | Protein | Threshold | Affected Region |
|----------------|----------------------|------------------|----------------------------|
| SBMA | Androgen Receptor | 38 | Motor neurons |
| HD | Huntingtin | 36 | Striatum, cerebral cortex |
| SCA1 | Ataxin 1 | 40 | Cerebellar Purkinje cells |
| SCA2 | Ataxin 2 | 32 | Cerebellar Purkinje cells |
| SCA3 | Ataxin 3 | 61 | Cerebellar dentate neurons |
| SCA6 | CACNA1a | 20 | Cerebellar Purkinje cells |
| SCA7 | Ataxin 7 | 37 | Cerebellum |
| SCA17 | TATA Binding Protein | 47 | Cerebellar Purkinje cells |
| DRPLA | Atrophin 1 | 49 | Cerebellum |

SBMA, spinobulbar muscular atrophy; HD, Huntington's disease, SCA, spinocerebellar ataxia; DRPLA, dentatorubropallidoluysian atrophy; CACNA1a, α_{1a} -voltage-dependent calcium channel

Chapter 2

Identification of Full-Length Androgen Receptor and Huntingtin as Actin-Binding Proteins

Introduction

The androgen receptor (AR) and huntingtin (Htt) protein do not appear to have much in common other than the presence of their polyglutamine tract. AR is a well-characterized nuclear transcription factor that binds to dihydrotestosterone (DHT) and mediates transcriptional effects on AR response elements (Beato and Klug, 2000; Quigley et al., 1995). It contains an N-terminal transactivation domain (TAD), a central DNA binding domain (DBD), and a C-terminal ligand-binding domain (LBD) (Fig. 1A) (Beato and Klug, 2000; Quigley et al., 1995). Htt is a large, cytoplasmic protein whose exact function is unclear (Fig. 2A). It has been implicated in transcriptional regulation, pro-survival functions, and vesicle integrity (Harjes and Wanker, 2003). Here, we have found that these proteins bind to filamentous actin (F-actin) and discuss the implications below.

Results

Full-Length AR Binds to F-actin in Cells.

To determine if there is an interaction between full-length AR (FL-AR) and F-actin, we performed a phalloidin-mediated coprecipitation similar to previously described methods (Fulga et al., 2007). Phalloidin, a drug that specifically binds to F-actin (dos Remedios et al., 2003), was used to selectively precipitate F-actin from cells. HEK293 cells were transiently transfected with either unexpanded (Q25) or expanded (Q65) FL-AR (Fig. 1A). After 24h, cells were treated with 2 μ M latrunculin or DMSO for 1h. Cells were then lysed in 1% Triton and exposed to a biotin-phalloidin conjugate. A pull-down was performed using streptavidin beads. Coprecipitated proteins were resolved by

western blot using antibody to the N-terminus of AR (N-20). Both unexpanded and expanded FL-AR were detected in the precipitate (Fig. 1B). Prior treatment of the cells with 2 μ M latrunculin eliminated FL-AR from the precipitate, indicating a specific interaction with F-actin (Fig. 1B). Immunostaining of HA-tagged FL-AR (Q25) in C17.2 neural precursor cells showed both cytoplasmic and nuclear distribution, with colocalization with F-actin predominantly at membrane edges (Fig. 1C). After treating cells with AR ligand, DHT, FL-AR (Q25) showed an exclusively nuclear distribution, and no colocalization with F-actin was detected (Fig. 1D).

Full-length Huntingtin Binds to F-actin via Cosedimentation Assay.

To determine if full-length huntingtin (FL-Htt) interacts with F-actin, HEK293 cell extracts containing FL-Htt were used in an F-actin cosedimentation assay. This assay was originally performed by Jieya Shao. FL-Htt is a very large protein (~350kDa) and has proven difficult to transiently express. Many smaller degradation products of Htt are observed after transient expression (Figure 2B, arrowheads). Cell extracts of FL-Htt were first precleared by ultracentrifugation (100,000 xg) and then incubated with F-actin that had been pre-polymerized *in vitro*. Mixtures were ultracentrifuged (100,000 xg), and supernatant and pellet fractions were resolved by western blot (for Htt) and Coomassie (for actin). F-actin can be seen strongly localizing to the pellet fraction, while there is no actin present in control samples (Fig. 2B). Likewise, FL-Htt can be seen partially cosedimenting with F-actin in the pellet fraction, while it remains completely soluble in its absence (Fig. 2B). Thus, FL-Htt appears to interact with F-actin.

Discussion

Implications for AR, Actin-Binding, and Transcriptional Activation.

There is growing evidence that proteins normally associated with cytoskeletal functions can also regulate steroid receptor activity. In particular, several actin-binding proteins have been described to act as AR coregulators (Archer et al., 2004; Archer et al., 2005; Loy et al., 2003; Nishimura et al., 2003; Ozanne et al., 2000; Ting et al., 2004; Ting et al., 2002; Truica et al., 2000; Yang et al., 2002). In addition to their actin-binding abilities, these proteins specifically bind AR and can translocate into the nucleus with the receptor. For example, filamin, an F-actin crosslinking protein, binds the hinge domain of AR, which connects the central DBD and LBD (Loy et al., 2003; Ozanne et al., 2000). Full-length filamin represses AR transactivation and a naturally occurring fragment of filamin translocates into the nucleus with AR (Loy et al., 2003; Ozanne et al., 2000). β -catenin, an adherens junction protein that links transmembrane cadherins to the actin cytoskeleton, binds the LBD of AR, enhances AR transactivation, and shows some translocation into the nucleus with AR after agonist induction (Truica et al., 2000; Yang et al., 2002). Intriguingly, huntingtin interacting protein 1 (HIP1), a protein originally found to bind huntingtin (Kalchman et al., 1997; Wanker et al., 1997), was found to bind AR, translocate with AR into the nucleus, and activate transcription in a ligand-dependant manner (Mills et al., 2005). Thus, both AR and Htt interact with HIP1 and F-actin, though the link to polyglutamine dysfunction, if any, remains to be explored.

Members of the gelsolin gene family share homologous actin-binding domains. At least two proteins in this family, gelsolin and supervillin, possess AR coactivator functions (Archer et al., 2004; Archer et al., 2005). Gelsolin, an actin severing and

capping protein, binds AR in the DBD and LBD (Nishimura et al., 2003). It enhances AR transactivation in a ligand-dependent manner and travels to the perinucleus with AR during nuclear translocation (Nishimura et al., 2003). Supervillin, an F-actin bundling protein, binds at several regions of AR (Ting et al., 2002). It enhances AR transactivation in a ligand-dependent manner and colocalizes with AR in the nucleus (Ting et al., 2002). Furthermore, the ability for supervillin to increase AR transactivation may depend on the availability of actin monomers (Ting et al., 2004). Thus, our finding that AR associates with F-actin from cell extracts is not surprising in light of these previous findings. The interaction may represent an indirect binding, or AR itself may possess binding sites for direct interaction. Understanding the normal function of actin in AR regulation opens an unexplored research avenue and may lead to a better understanding of steroid regulation.

Implications for Htt, Actin-Binding, and Normal Function.

Though the exact function of huntingtin is not clear, researchers have inferred its role from the many interaction partners that have been identified. Many of these interaction partners are closely associated with actin or actin dynamics. Though the interactions between Htt and actin-related proteins are vast, they are also inconclusive. Htt interacts with many proteins that regulate clathrin-mediated endocytosis (Harjes and Wanker, 2003), which requires the actin-cytoskeleton to pinch off vesicles (McPherson, 2002; Qualmann and Kessels, 2002). Huntingtin interacting protein 1, or HIP1, is a mammalian ortholog of Sla2, which control actin assembly in yeast (Holtzman et al., 1993). HIP1 can bind to membranes, actin, and clathrin. Htt and HIP1 together can bind

to a subunit of AP-2, a protein important in initiating clathrin-mediated endocytosis. Thus, Htt and HIP1 may work in concert with many cofactors to regulate clathrin-mediated endocytosis (Engqvist-Goldstein et al., 1999; Legendre-Guillemain et al., 2002; Senetar et al., 2004; Waelter et al., 2001). Htt mediates many interactions with proteins containing SH3-domains via its polyproline-rich domain (adjacent to the polyglutamine tract) (Harjes and Wanker, 2003). One such protein is PACSIN (Modregger et al., 2002), a protein also previously implicated in endocytosis (Modregger et al., 2000; Qualmann and Kelly, 2000; Qualmann et al., 1999; Wasiak et al., 2001). PACSIN can bind to N-WASP, an upstream regulator of F-actin formation (Modregger et al., 2000; Qualmann and Kelly, 2000; Qualmann et al., 1999; Wasiak et al., 2001), implicating a role for Htt in actin-assembly. Htt binds to SH3GL3 via its polyproline domain (Sittler et al., 1998), possibly to regulate vesicle release in concert with dynamin and synaptojanin (Hill et al., 2001; Ringstad et al., 1997). Profilin, a canonical G-actin binding protein (Witke, 2004), likely binds to Htt via its polyproline-rich domain (Goehler et al., 2004; Shao et al., 2008b). Emerging evidence has linked profilin to endocytosis via proteins like dynamin, again implicating a role for Htt in vesicle release (Gareus et al., 2006; Witke et al., 1998).

Htt interacts with many proteins linked to GTPase signaling, upstream molecules important for the regulation of actin-assembly (Mullins, 2000). Htt binds postsynaptic density protein, PSD-95, which has been shown to associate with a Ras-GTPase activating protein (Kim et al., 1998) and a Rac guanine-nucleotide exchange factor (Penzes et al., 2001). Htt interacts with Cdc42 interacting protein 4 (CIP4) (Holbert et al., 2003), ADP ribosylation factor (ARF)-interacting protein (arfaptin 2) (Peters et al., 2002), and tumor-necrosis factor- α inducible coiled-coiled protein (FIP-2) (Hattula and

Peranen, 2000), all GTPase-interacting proteins. It also interacts with a G-protein coupled receptor kinase interacting protein (GIT1) (Goehler et al., 2004), another integrator of cytoskeletal information. Thus, Htt appears to be a central regulator of information that may eventually lead to actin-cytoskeletal remodeling. Our data showing a specific interaction between Htt and F-actin (Fig. 2) may simply reflect an indirect association via the many intermediate actin-binding proteins, or they may imply a more direct role for Htt in cytoskeletal remodeling. In either case, a better understanding of Htt's normal function will likely be beneficial to understanding polyglutamine dysfunction.

Material and Methods

Cell Culture and Transfection. For 12-well cell-culture dishes, HEK293 cells were plated at 650,000 cells/well and transfected with .6 μ g total DNA (ps6R-FLARQ25 or Q65) with Lipofectamine and Plus reagent (Invitrogen) according to the manufacturer's instructions. C-17.2 cells were plated at 100,000 cells per 24-well and transfected with .6 μ g DNA with Lipofectamine 2000 (Invitrogen) according to the manufacturers instructions.

Phalloidin Coprecipitation. 24h after transfection, cells were treated with either 2 μ M latrunculin A (Molecular Probes) or DMSO for 2 hours prior to harvesting. Cell pellets were lysed cells in 50 μ l lysis buffer (PBS, 5mM EDTA, 1% triton, protease inhibitor cocktail (Roche)) and diluted in up to 200 μ l resuspension buffer (PBS, 5mM EDTA). 2.5 μ l of 100Units/ml of biotin-XX-phalloidin (Molecular Probes) was added to lysates for 1H at 4°Celsius. Lysates were then added to 25 μ l of washed M-280 streptavidin

dynabeads (Invitrogen) for 1H at 4° Celsius. Beads were washed 3X in resuspension buffer plus .1% Tween. Beads were boiled for 5 minutes in 100µl 1X SDS sample buffer plus 25mM DTT and subjected to SDS-PAGE. Western blots were used to detect co-precipitated proteins and Coomassie stain was used to detect actin.

Confocal Microscopy. All images were acquired on a C1sl confocal microscope (Nikon Instruments Inc.).

Immunofluorescence. C17.2 cells were mounted on polyornithine-coated glass coverslips 48 hours after transfection at a density of 20,000 cells per coverslip for 60X imaging. Cells were fixed in 4% paraformaldehyde, treated with .5% Triton, and blocked in 5% BSA for 1 hour. Coverslips were treated with HA antibody (1:500, Covance) overnight at 4° C, rinsed 4X and washed 3X with %1 TBS-Tween, and treated with donkey-anti-mouse Alexa-Fluor 488 (1:400, Molecular probes) for 1h at 37°C. Coverslips were rinsed 4X and washed 2X with %1 TBS-Tween. F-actin was visualized with rhodamine-conjugated phalloidin (1:300, Molecular Probes) and the nucleus was stained with DAPI (Sigma). Coverslips were mounted with anti-fade mounting media (Invitrogen) and analyzed 24h later.

F-actin Co-sedimentation. Non-muscle human actin (Cytoskeleton) was polymerized according to the manufacturer's instructions. 10 mg/ml of G-actin was polymerized with polymerization buffer (10X: 500mM KCl, 20mM MgCl₂, 10mM ATP) in general actin buffer (5mM Tris-HCl pH8.2mM CaCl₂, .5mM DTT, .2mM ATP) for 1h at RT. Equal

molar ratios of unlabeled phalloidin (Molecular Probes) were added to stabilize filaments. Cell extracts were precleared via ultracentrifugation (100,000 x g for 30min at 4°C). Lysates were added to a pre-polymerized F-actin (4uM) or a BSA control for 1h on ice. Mixtures were ultracentrifuged for 30min at 100,000 x g at 4°C. Supernatants and pellets were subjected to SDS-PAGE. F-actin pellets were visualized via Coomassie stain. Htt was visualized by antibody.

Antibodies. Anti-rabbit N-20 antibody (1:1000, Santa Cruz) was used for the detection of FL-AR products.

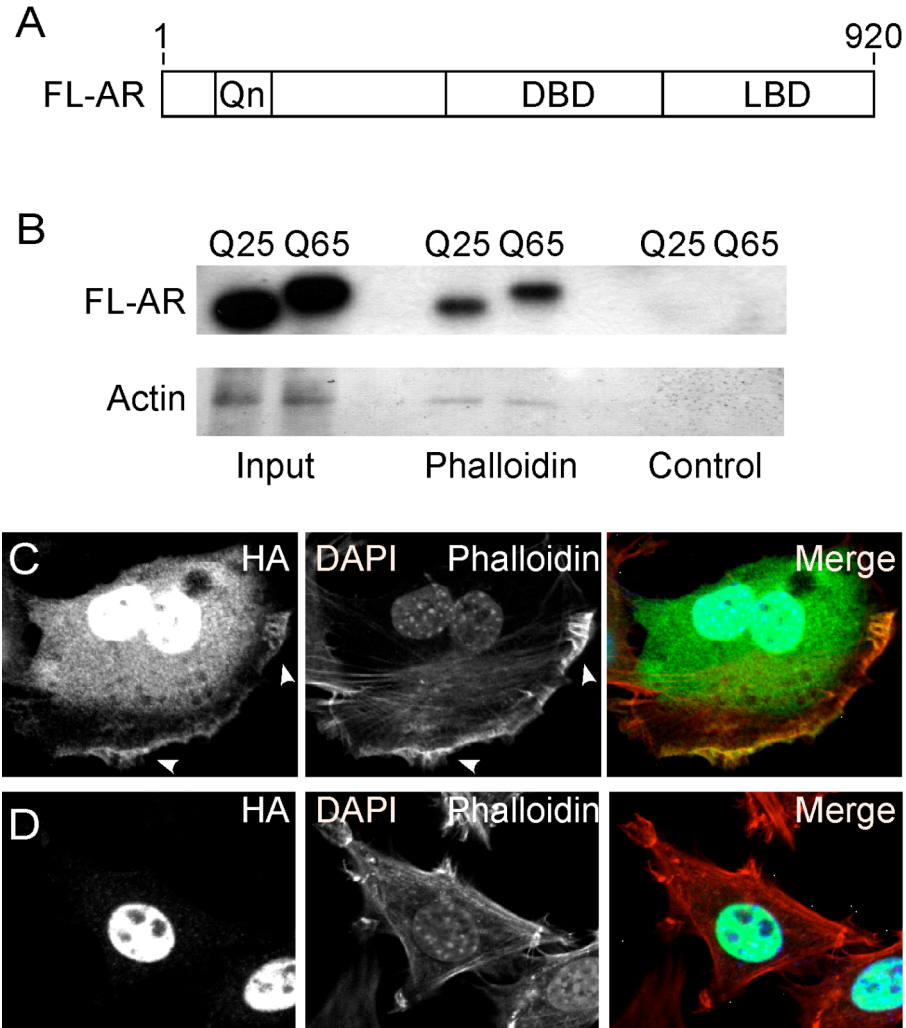


FIGURE 1: Full-length AR binds to F-actin in cells

A, Schematic of full-length AR. Qn, polyglutamine tract. DBD, DNA-binding domain. LBD, Ligand-binding domain. *B*, FL-AR binds to F-actin from cell lysates. F-actin was precipitated with biotinylated-phalloidin from HEK293 cell lysates transiently expressing FL-AR with 25 or 65 glutamines. Control cells were pretreated with 2 μ M latrunculin A. Coprecipitates were probed via immunoblot. *C*, HA-immunofluorescent staining of FL-AR without DHT in C17.2 cells. FL-AR shows both nuclear and cytoplasmic localization. Colocalization with F-actin can be seen predominantly at membrane edges (arrowheads). *D*, HA-immunofluorescent staining of FL-AR with DHT in C17.2 cells. Cells were pretreated with 100nM DHT for 24 hours. FL-AR shows exclusive nuclear localization.

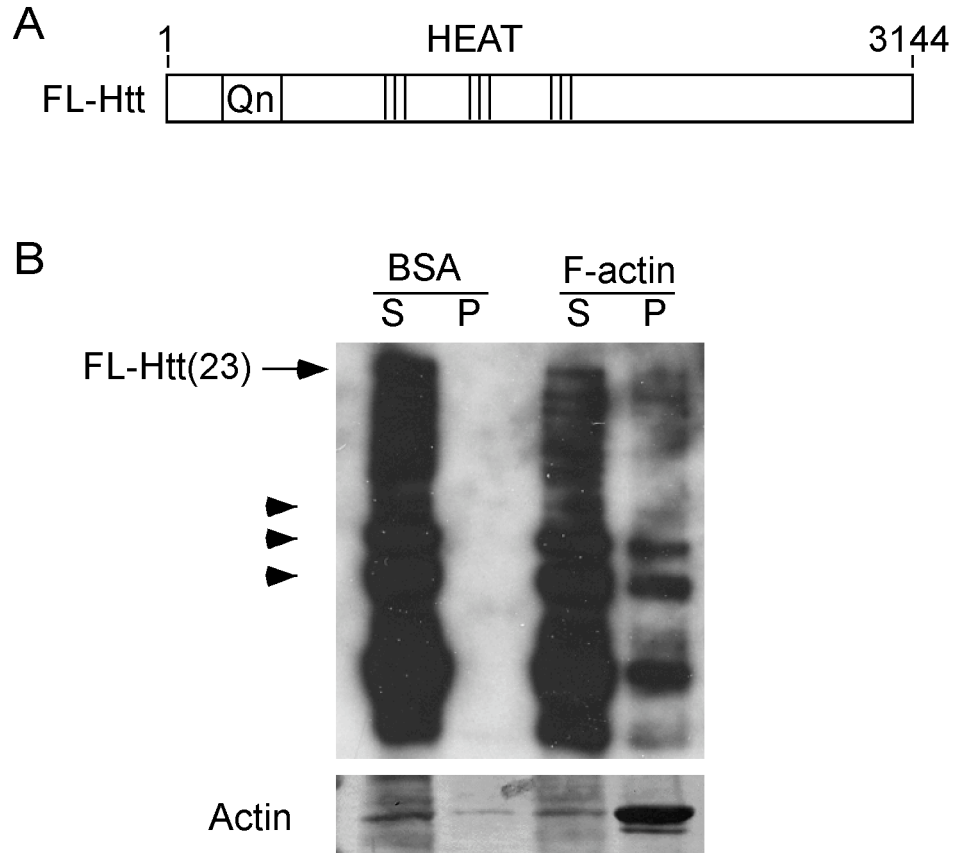


FIGURE 2: Full-length Htt binds to F-actin from cell extracts

A, Schematic of full-length Htt (FL-Htt). Qn, polyglutamine tract. HEAT (Huntingtin, Elongation factor 3, protein phosphatase 2A, TOR1). *B*, FL-Htt binds to F-actin from cell extracts. HEK293 cell lysates transiently expressing FL-Htt with 23 glutamines were incubated with pre-polymerized F-actin *in vitro*. Smaller degradation products of Htt can be seen (arrowheads). As control, lysates were mixed with BSA. Mixtures were ultracentrifuged and supernatant and pellet fractions were analyzed via immunoblot (Htt) or Coomassie (actin). Htt cosediments with F-actin in pellet fraction, while remaining soluble in its absence.

Chapter 3

F-actin Binding Regions on the Androgen Receptor and Huntingtin Increase Aggregation and Alter Aggregate Characteristics

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**F-actin Binding Regions on the Androgen Receptor and Huntingtin Increase
Aggregation and Alter Aggregate Characteristics**

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Abstract

Protein aggregation is associated with neurodegeneration. Polyglutamine expansion diseases such as spinobulbar muscular atrophy and Huntington disease feature proteins that are destabilized by an expanded polyglutamine tract in their N-termini. It has previously been reported that intracellular aggregation of these target proteins, the androgen receptor (AR) and huntingtin (Htt), is modulated by actin-regulatory pathways. Sequences that flank the polyglutamine tract of AR and Htt might influence protein aggregation and toxicity through protein-protein interactions, but this has not been studied in detail. Here we have evaluated an N-terminal 127 amino acid fragment of AR and Htt exon 1. The first 50 amino acids of ARN127 and the first 14 amino acids of Htt exon 1 mediate binding to filamentous actin *in vitro*. Deletion of these actin-binding regions renders the polyglutamine-expanded forms of ARN127 and Htt exon 1 less aggregation-prone, and increases the SDS-solubility of aggregates that do form. These regions thus appear to alter the aggregation frequency and type of polyglutamine-induced aggregation. These findings highlight the importance of flanking sequences in determining the propensity of unstable proteins to misfold.

Introduction

Spinobulbar muscular atrophy (SBMA) and Huntington disease (HD) are devastating neurodegenerative diseases. SBMA is caused by an expanded CAG trinucleotide repeat that encodes a long polyglutamine tract in the androgen receptor (AR) (La Spada et al., 1991), while HD is caused by an enlarged polyglutamine tract in the huntingtin (Htt) protein (1993). Proteolytic cleavage of AR and Htt appears to

generate toxic, N-terminal fragments (Goldberg et al., 1996; Li et al., 2007; Li et al., 1998; Wellington et al., 1998). These are sufficient to recapitulate neurodegenerative phenotypes *in vivo* (Abel et al., 2001; Davies et al., 1997). N-terminal fragments of expanded AR and Htt readily aggregate *in vitro* and in cell-culture models, thus making them useful in biochemical studies (Merry et al., 1998; Pollitt et al., 2003; Scherzinger et al., 1997). While the aggregation and toxicity of polyglutamine proteins directly correlate with the length of the polyglutamine tract (Scherzinger et al., 1997), flanking sequences are also clearly important (Bhattacharyya et al., 2006; Duennwald et al., 2006b; Thakur et al., 2009), as are intracellular signaling pathways that act via protein interactions or post-translational modifications (Diamond et al., 2000; Humbert et al., 2002; Pollitt et al., 2003). Emerging evidence from a variety of studies of aggregation-prone proteins associated with neurodegenerative diseases suggests that there is considerable diversity among aggregates that can be formed *in vitro* (Frost et al., 2009; Muchowski et al., 2000; Nekooki-Machida et al., 2009), and, moreover, that some protein aggregates are likely to be more toxic than others (Nekooki-Machida et al., 2009; Petkova et al., 2005). Thus, protein interactions that alter aggregate conformation could play an important role in determining toxicity.

Indirect evidence implicates actin and/or actin-binding factors as an influence on polyglutamine-dependent aggregation of AR and Htt (Bauer et al., 2009; Burnett et al., 2008; Meriin et al., 2007; Meriin et al., 2003; Pollitt et al., 2003; Shao et al., 2008a; Shao et al., 2008b; Suhr et al., 2001). Y-27632, a rho-kinase (ROCK) inhibitor, reduces intracellular polyglutamine aggregation of Htt exon 1 and the N-terminal fragment of AR, termed ARN127 (Pollitt et al., 2003; Shao et al., 2008a). Y-27632 also attenuates Htt

toxicity in *Drosophila* and improves motor function in mice (Li et al., 2009; Pollitt et al., 2003). Y-27632 blocks phosphorylation of profilin, an actin-binding protein that directly binds Htt, but not AR (Goehler et al., 2004; Shao et al., 2008b). Profilin strongly inhibits aggregation of ARN127 and Htt exon 1 in cells (Shao et al., 2008b), and decreases polyglutamine-mediated toxicity in *Drosophila* (Burnett et al., 2008). The anti-aggregation effects of profilin depend on both its polyproline binding activity (required to bind Htt), and its ability to bind G-actin (required to suppress both Htt and AR aggregation) (Shao et al., 2008b). In this study, we have identified regions of ARN127 and Htt exon 1 that bind filamentous actin (F-actin) *in vitro*, and investigate the effect of these regions on polyglutamine-dependent aggregation.

Results

ARN127 and Htt exon 1 bind F-actin in vitro.

To test for a direct interaction between AR, Htt, and F-actin *in vitro*, we used an F-actin co-sedimentation assay with recombinant GST-ARN127 or GST-Htt exon 1 containing 25 glutamine repeats (Fig. 1A). Coomassie staining confirmed protein purity (Fig. S1A,E). Protein preparations were precleared by ultracentrifugation to remove any pre-existing aggregates. 0.5 μ M GST-ARN127(25) or 0.25 μ M GST-Htt exon 1(25) was incubated with F-actin (4 μ M) that had been pre-polymerized *in vitro* for 1 hour at 25° C. As a control, proteins were incubated with an equal concentration of bovine serum albumin (BSA) instead of F-actin. After ultracentrifugation (100,000 x g), supernatant and pellet fractions were analyzed by western blot using antibody to GST. Western blot analysis was used rather than Coomassie stain because both proteins are very similar in

size to G-actin (43 kDa). F-actin localized to the pellet fraction in all cases, as visualized by Coomassie stain (Fig. 1B). Both GST-ARN127(25) and GST-Htt exon 1(25) cosedimented with F-actin while remaining soluble in its absence (Fig. 1B). GST alone did not co-sediment with F-actin (Fig. 1B). ARN127 also bound F-actin following cleavage of the GST-tag (Fig. S1B). We determined the binding affinity of GST-ARN127(25) and GST-Htt exon 1(25) for F-actin by using a constant amount of protein (16nM for ARN127, 150nM for Htt exon 1) mixed with increasing amounts of F-actin (Fig. 1C, D). GST-ARN127(25) bound to F-actin with an approximate dissociation constant (K_d) of 1 μ M (Fig. 1C), while for GST-Htt exon1(25) the K_d was approximately 2 μ M (Fig. 1D), comparable to other actin-binding proteins (dos Remedios et al., 2003).

Amino acids 1-50 of ARN127 and 1-14 of Htt exon 1 mediate F-actin binding.

We used deletion analysis to map the actin-binding regions of ARN127 and Htt exon 1 (Fig. 2A,C). Protein purity was assessed by Coomassie (Fig. S1A,C,E). Deletion of the polyglutamine domain of ARN127 and peptides C-terminal to the polyglutamine domain (AR₁₋₅₇) had no appreciable effect on actin binding (Fig. 2B), while deleting the N-terminal peptides of AR (AR₅₀₋₁₂₇, AR₇₈₋₁₂₇) abolished binding (Fig. 2B). Constructs lacking the polyglutamine tract (ARN127(Δ Q)), or containing an expanded polyglutamine tract (ARN127(52)) bound actin equivalently (Fig. 2B). A very pure extract of AR₁₋₅₇ cleaved from the GST-tag also bound F-actin efficiently (Fig. S1D). Deletion of peptides C-terminal to the polyglutamine tract of Htt exon 1 (Htt₁₋₄₅) had no effect on binding while deletion of the first 14 amino acids (Htt₁₅₋₉₂) abolished binding (Fig. 2C,D). Thus, the first 50 amino acids (N50) of AR and the first 14 amino acids

(N14) of Htt mediate interactions with F-actin *in vitro*. There is no apparent homology between these regions.

Sensitivity to actin-regulatory pathways requires the first 50 amino acids of ARN127.

We have previously found that Y-27632, as well as a downstream ROCK target, profilin, inhibit ARN127 and Htt exon 1 aggregation (Pollitt et al., 2003; Shao et al., 2008a; Shao et al., 2008b). Since both Y-27632 and profilin regulate actin assembly (Amano et al., 2000; Witke, 2004), we tested whether deletion of the actin-binding region of ARN127 would alter its response to their inhibitory activities. We employed fluorescence resonance energy transfer (FRET) to quantify these effects (Pollitt et al., 2003). We were only able to test AR using this assay, since deletion of the N14 region of Htt prevented significant FRET, possibly due to altered orientation of the CFP and YFP moieties. Expanded ARN127 and ARQC with 65 glutamines were tagged with fluorescence donor or acceptor tags, CFP or YFP, and co-transfected into HEK293 cells as previously described (Pollitt et al., 2003). Y-27632 decreased ARN127(65)-CFP/YFP aggregation dose-dependently (Fig. 3A). Intriguingly, it increased the aggregation of ARQC(65)-CFP/YFP (Fig. 3A). Similarly, profilin 1 dose-dependently reduced ARN127(65)-CFP/YFP aggregation, but was much less effective on ARQC(65)-CFP/YFP aggregation (Fig. 3B). Thus, the N50 region of AR mediates effects of these actin regulators on polyglutamine-mediated aggregation.

N50 of ARN127 affects inclusion type, number, and distribution.

To better characterize the behavior of expanded AR and Htt peptides within the cell, and to determine the influence of the actin-binding regions, we transfected expanded forms of each construct into C17.2 neural precursor cells, which were used for their ease of imaging. Identical results were obtained with HEK293 cells (data not shown). We observed clear differences between inclusions formed by ARN127(65)-YFP vs. ARQC(65)YFP. ARN127(65)-YFP formed many different types of inclusions (single, multiple, nuclear, cytoplasmic), whereas ARQC(65)YFP tended to form one perinuclear inclusion (Fig. 4A-F). After 48h post-transfection, ARN127(65)-YFP expression resulted in multiple inclusions per cell in ~57% of cells vs. ~23% for ARQC(65)-YFP (Fig. 4G). ARN127(65)-YFP produced nuclear inclusions in ~19% of cells vs. ~5% of cells for ARQC(65)-YFP (Fig. 4H). These data are consistent with the idea that protein interactions mediated by the N50 domain of AR could alter the type of protein aggregates, as well as their subcellular localization. In contrast, we did not observe obvious inclusion differences between Htt exon 1 and HttQC. Htt exon 1(72)-YFP and HttQC(72)-YFP formed inclusions with similar visual characteristics (data not shown) as did Htt exon 1(97)-H4 and HttQC(97)-H4 (Fig. 4I-N), which contain a HIS-HA-HA-HIS epitope tag (Thompson et al., 2009) (Fig. 5B).

N50 of ARN127 and N14 of Htt exon 1 promote inclusion formation.

To quantify the effect of N50 of ARN127 and N14 of Htt exon 1 on intracellular aggregation, we transiently transfected HEK293 cells with expanded AR constructs (ARN127(65)-YFP or ARQC(65)-YFP) or Htt constructs (Htt exon 1(72)-YFP or HttQC(72)-YFP; Htt exon 1(97)-H4 or HttQC(97)-H4, which contain a HIS-HA-HA-HIS

epitope tag) and cultured the cells for 24h (Fig. 5A-E). In each case, the QC proteins produced significantly fewer visible inclusions (Fig. 5C-E). Biochemical analysis of total cell lysates after 24h in 2% SDS revealed that the soluble fractions of both ARQC(65)-YFP and HttQC(72)-YFP were enriched, while the SDS-insoluble aggregates, were depleted (Fig. 5F-H). Thus, N50 of ARN127 and N14 of Htt exon 1 each promote the formation of inclusions and SDS-insoluble aggregates.

N50 of ARN127 and N14 of Htt exon 1 promote SDS-insoluble aggregates.

The distinct effects of actin-binding regions on AR and Htt inclusion formation suggested that they might also change aggregate characteristics. Thus, we transfected HEK293 cells with the various constructs as above. 24h post-transfection the cells were disrupted via syringe lysis and fractionated by centrifugation (15,000 x g) to compare soluble vs. insoluble species. Supernatant and pellet fractions were resuspended in 2% SDS and resolved by SDS-PAGE and western blot. All protein in the supernatant fraction was completely solubilized by SDS (Fig. 6A). In contrast, both ARN127(65)-YFP and ARQC(65)-YFP peptides that partitioned into the insoluble pellet contained mixtures of SDS-soluble and insoluble proteins (Fig. 6A,B). The aggregates of ARQC(65)-YFP were much more readily dissociated in 2% SDS compared to ARN127(65)-YFP (Fig. 6A,B). Parallel experiments in C17.2 cells revealed similar results (Fig. 6A). We observed similar phenomena for Htt exon 1(97)-H4 vs. HttQC(97)-H4 (Fig. 6C). The pellet fraction containing HttQC(97)-H4 was significantly more SDS-soluble (Fig. 6C,D). Thus flanking sequences of AR and Htt influence both the propensity for protein misfolding and the biochemical characteristics of the aggregates that result.

Discussion

In this study, we have focused on aggregation-prone fragments of AR (ARN127) and Htt (Htt exon 1), identifying amino acids 1-50 (N50) of ARN127 and 1-14 (N14) of Htt exon1 as regions that influence polyglutamine-dependent aggregation. These regions were originally identified because they mediate binding to F-actin *in vitro*. However, we did not observe co-localization of ARN127 or Htt exon 1 with the F-actin cytoskeleton (data not shown). This could reflect lack of significant binding, or that intracellular binding to F-actin is transient. Deletion of the N50 region of ARN127 or the N14 region of Htt decreased the total number of inclusions formed, and the aggregates that did form were more SDS-soluble. Further, the N50 region of AR regulates the characteristics of inclusions formed and mediates its responsiveness to the anti-aggregation effects of Y-27632 and profilin. Thus, the flanking sequence of the polyglutamine region does not simply alter the propensity to form a polyglutamine aggregate, but can directly affect its aggregation rate and responses, the subcellular localization of the inclusions, and the biochemical characteristics of the aggregates.

The importance of flanking sequences on Htt exon 1 has previously been demonstrated: flanking sequences have been shown to directly regulate aggregation, toxicity, and the morphology of inclusions (Duennwald et al., 2006b). Given the importance of primary amino acid sequence on polyglutamine aggregation *in vitro* (Bhattacharyya et al., 2006; Thakur et al., 2009), we cannot exclude that the intracellular aggregation differences observed are solely due to altered intrinsic aggregation kinetics. However, this cannot explain all observable differences noted, such as altered distribution of polyglutamine inclusions. We have found that peptides containing either the first 50

amino acids of AR or the first 14 amino acids of Htt partition to Triton-insoluble cell fractions independent of their inherent solubility (Figs. S2, S3), suggesting that these regions could mediate protein interactions with macromolecular structures in the cytoplasm. Indeed, the first 17 amino acids of Htt have previously been implicated as a pro-aggregation-domain, a cytoplasm-targeting domain, and have been shown to bind mitochondria, the endoplasmic reticulum, and the Golgi apparatus (Atwal et al., 2007; Rockabrand et al., 2007). This region is highly conserved across diverse species. Taken together, these studies imply that this region is likely to mediate protein interactions that play an important role in determining aggregation properties of the Htt peptide.

Although we did not detect binding of AR or Htt to F-actin in cells, the *in vitro* binding of the Htt N-terminus to F-actin is intriguing in light of Htt's capacity also to bind the actin remodeling factor profilin. Profilin/Htt interaction is most likely mediated by Htt's polyproline domain, immediately distal to the polyglutamine region in the Htt peptide (Goehler et al., 2004; Shao et al., 2008b). Analysis of the first 17 amino acids of Htt reveal a striking similarity to another actin-binding peptide, Lifeact (Riedl et al., 2008). These observations may indicate a normal, perhaps transient, function for Htt in the regulation of the actin cytoskeleton, although this remains to be tested.

In this study, we find that sequences independent of the polyglutamine tract have profound effects on subcellular localization, detergent solubility, and inclusion formation of polyglutamine peptides. Given the apparent importance of these regions for protein interaction, this implies that modifiers of protein aggregation are likely to be found within the set of AR and Htt interacting proteins. Indeed, the recent finding that Htt-interacting proteins are enriched for genetic modifiers of toxicity is consistent with this idea

(Kaltenbach et al., 2007). Further study of AR and Htt binding proteins thus may reveal mechanisms that specify particular aggregation pathways and pathogenic features of each disease.

Materials and Methods

Constructs. Bacterial expression vectors for ARN127 Htt exon 1 and various derivatives were constructed via PCR amplification and subcloned in the pGEX4T1 backbone (Amersham Biosciences). Similarly, for mammalian expression vectors, ARQC and HttQC plasmids were constructed via PCR amplification and subcloned into pECFP-N1, pEYFP-N1 (Clontech), or pcDNA.3 backbones. GST-Htt exon 1 constructs were originally obtained from Paul Muchowski and mammalian pcDNA3-Htt-H4 constructs were originally obtained from Joan Steffan.

Cell Culture and Transfection. HEK293 cells were plated at 250,000 cells per well in a 24-well dish and transfected with .3 μ g total DNA with Lipofectamine and Plus reagent (Invitrogen) according to the manufacturer's instructions. C-17.2 cells were plated at 100,000 cells per 24-well and transfected with .6 μ g DNA with Lipofectamine 2000 (Invitrogen) according to the manufacturers instructions. Cells were harvested at indicated times.

FRET. All FRET measurements were carried out 48 hours after transfection of HEK293 cells, read in 96-well cell-culture plates by a fluorescence plate reader (Tecan). HEK293 cells were transfected with .075 μ g total ARN127CFP/YFP DNA in a 1:3 donor:acceptor

ratio. Profilin DNA was co-transfected with AR constructs at concentrations of .075 μ g, .15 μ g, or .225 μ g. pcDNA3 backbone vector was also co-transfected for a constant final concentration of .3 μ g. Y-27632 was added at the indicated concentration for 24h prior to FRET measurements[29].

Confocal Microscopy. All images were acquired on a C1sl confocal microscope (Nikon Instruments Inc.).

Immunofluorescence. C17.2 cells were mounted on polyornithine-coated glass coverslips 48 hours after transfection at a density of 20,000 cells per coverslip for 60X imaging. Cells were fixed in 4% paraformaldehyde, treated with .5% Triton, and blocked in 5% BSA for 1 hour. Coverslips were treated with HA antibody (1:500, Covance) overnight at 4° C, rinsed 4X and washed 3X with %1 TBS-Tween, and treated with donkey-anti-mouse Alexa-Fluor 488 (1:400, Molecular probes) for 1h at 37°C. Coverslips were rinsed 4X and washed 2X with %1 TBS-Tween. F-actin was visualized with rhodamine-conjugated phalloidin (1:300, Molecular Probes) and the nucleus was stained with DAPI (Sigma). Coverslips were mounted with anti-fade mounting media (Invitrogen) and analyzed 24h later.

Inclusion Counting. At least 100 HEK293 cells per transfection (3) were examined for total number of inclusions formed after 24h. Approximately 100 C17.2 cells per transfection (3) were counted for inclusions. Cells were identified as having nuclear, cytoplasmic, or multiple inclusions, or a combination.

Protein Purification. GST-ARN127 plasmids were grown in *E. Coli* Rosetta 2 (DE3) competent cells (Novagen). Protein expression was induced with 1mM (isopropyl β -D-thiogalactoside) IPTG for 3h at 37°C. GST-Htt plasmids were grown in *E. Coli* SURE competent cells (Stratagene). Protein expression was induced with 1mM IPTG for 3H at 30°C. Bacterial pellets were resuspended in resuspension buffer (PBS, .05% Tween, 1mM PMSF, protease inhibitor tablet (Roche)) and lysed by sonication and 1% Triton. GST-tagged proteins were precipitated with glutathione sepharose (Amersham Biosciences) and eluted with equal volumes of elution buffer (50mM Tris-HCl pH. 8m 10mM reduced glutathione). For cleavage of the GST-tag, 37.5 units of thrombin protease (Amersham Biosciences) was used for .5ml of GST-bound glutathione sepharose 4B (Amersham Sepharose) at 4°C overnight. Thrombin was removed from cleaved AR with benzamidine sepharose 6B (Amersham Biosciences). Protein concentration was quantified via Bradford assay and Coomassie staining via Image J.

F-actin Co-sedimentation. Non-muscle human actin (Cytoskeleton) was polymerized according to the manufacturer's instructions. 10 mg/ml of G-actin was polymerized with polymerization buffer (10X: 500mM KCl, 20mM MgCl₂, 10mM ATP) in general actin buffer (5mM Tris-HCl pH8.2mM CaCl₂, .5mM DTT, .2mM ATP) for 1h at RT. Equal molar ratios of unlabeled phalloidin (Molecular Probes) were added to stabilize filaments. Recombinant, purified GST-tagged proteins were precleared via ultracentrifugation (100,000 x g for 30min at 4°C). Proteins were added to a pre-polymerized F-actin (4uM) or a BSA control for 1h on ice. Mixtures were ultracentrifuged for 30min at 100,000 x g at 4°C. Supernatants and pellets were subjected to SDS-PAGE. F-actin pellets were

visualized via Coomassie stain. GST-proteins were probed via western blot using GST antibody (Santa Cruz Bioscience).

Detergent Fractionation. For unexpanded AR and Htt constructs, HEK293 cells were harvested 24h post-transfection. Cell pellets were lysed in 130 μ l cold lysis buffer (PBS, 1 % Triton, 5mM EDTA, protease inhibitor cocktail (Roche)) and subjected to high-speed ultracentrifugation (100,000 x g). For expanded AR and Htt constructs, HEK293 cells were harvested 24h later post-transfection. Cell pellets were lysed by syringe passage in cold resuspension buffer (PBS, 5mM EDTA, protease inhibitor cocktail (Roche)) and subjected to tabletop centrifugation (15,000 x g). Supernatant and pellets fractions were denatured in 2% SDS buffer, 25mM DTT, and boiled for 10min. For crude cell-lysates, cell pellets were directly lysed in 200 μ l of 2% SDS, 25mM DTT and boiled for 10 minutes. All lysates were subjected to SDS-PAGE and probed via western blot using GFP antibody (Santa Cruz Biotechnology) or HA antibody (Covance).

Supernatants, pellets and higher molecular weight bands were quantified using Image J.

Antibodies. Anti-rabbit N-20 antibody (1:1000, Santa Cruz) was used for the detection of cleaved AR products. Anti-rabbit GFP-antibody (Santa Cruz) or anti-mouse HA-antibody (Covance) was used for the detection of YFP-tagged or HA-tagged proteins at 1:2000 dilution for soluble proteins or 1:1000 dilution for insoluble higher-molecular weights. Anti-mouse MW7 was used to detect the C-terminus of Htt at 1:2000 dilution for soluble proteins or 1:1000 dilution for insoluble higher-molecular weights.

Anti-mouse GST-antibody (1:2000, Santa Cruz) was used for the detection of GST-tagged proteins.

In Vitro Solubility.

GST-ARN127(25), GST-ARNQ(32), GST-ARQC(36), GST-Htt exon 1(25), and GST-HttQC(25) were recombinantly purified and quantified as described above. 1.25mg/ml of GST-AR or .75mg/ml of GST-Htt purified proteins were ultracentrifuged (100,000 x g) and incubated at 37° C for 1h to promote misfolding. For cleavage of the GST tag, 1.0 mg/ml of precleared GST-AR peptide were incubated with 1 NIH unit of thrombin (Invitrogen) overnight at 4° C. Proteins were ultracentrifuged and supernatant and pellet fractions were resuspended in SDS sample buffer and subjected to SDS-PAGE. Proteins were analyzed via Coomassie stain.

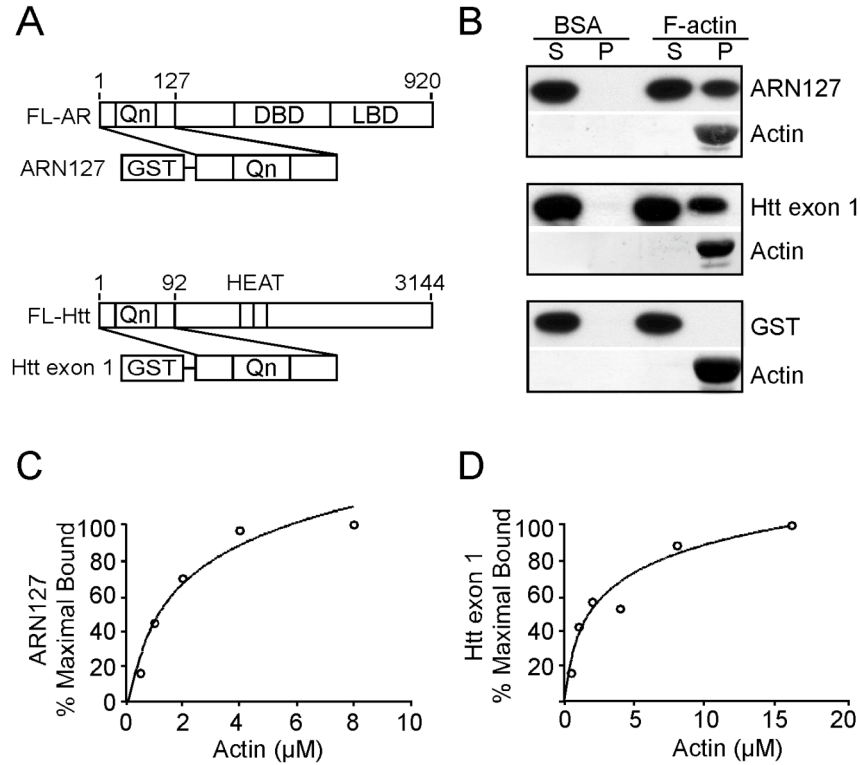


Figure 1: ARN127 and Htt exon 1 directly bind to F-actin *in vitro*.

A, Schematic of GST-tagged N-terminal fragments of AR and Htt, GST-ARN127 and GST-Htt exon 1, in comparison to full-length AR (FL-AR) and full-length Htt (FL-Htt). *B*, GST-ARN127(25) and GST-Htt exon 1(25) co-sediment with F-actin *in vitro* but remain soluble in the absence of F-actin. 0.5 μM of precleared GST-ARN127(25) or .25 μM of precleared GST-Htt exon 1(25) was mixed with 4 μM of pre-polymerized F-actin. Mixtures were ultracentrifuged at 100,000 x g and supernatant and pellet fractions were analyzed via western blot (ARN127 and Htt exon 1) and Coomassie (actin). GST alone does not co-sediment with F-actin. *C*, Binding affinity of GST-ARN127(25) to F-actin. The F-actin co-sedimentation assay was performed with 0, .5 μM, 1 μM, 2 μM, 4 μM, and 8 μM F-actin and .016 μM GST-ARN127(25). Gels were quantified to determine the percent bound to F-actin. Percent maximal binding is reported. *D*, Binding affinity of GST-Htt exon 1(25) to F-actin. The F-actin co-sedimentation assay was performed with 0, .5 μM, 1 μM, 2 μM, 4 μM, 8 μM, and 16 μM F-actin and .15 μM GST-Htt exon 1(25). Gels were quantified to determine the percent bound to F-actin using Image J.

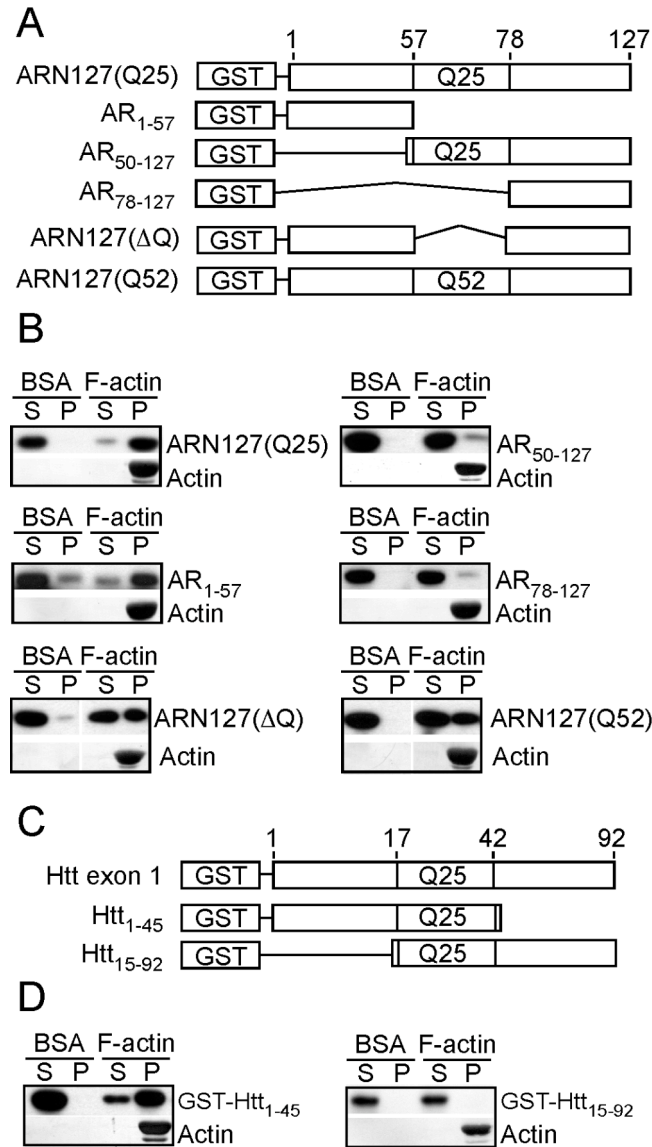


Figure 2: N50 of ARN127 and N14 of Htt exon 1 mediate F-actin binding *in vitro*.

A, Schematic of GST-ARN127 and various truncation mutants. *B*, The N-terminus of ARN127 binds to F-actin *in vitro*. GST-tagged truncations of ARN127(25) (AR₁₋₅₇, AR₅₀₋₁₂₇, AR₇₈₋₁₂₇ (20nM)), were tested for binding to F-actin (4μM). ARN127(25) and AR₁₋₅₇ co-sediment with F-actin while AR₅₀₋₁₂₇ and AR₇₈₋₁₂₇ do not. GST-tagged ARN127(ΔQ) or ARN127(52) (0.5μM) both co-sediment with F-actin. *C*, Schematic of GST-Htt exon 1 and GST-tagged truncation mutants. *D*, The N-terminus of Htt exon 1 binds to F-actin *in vitro*. GST-tagged Htt₁₋₄₅ (0.1μM) binds to F-actin (4μM) while Htt₁₅₋₉₂ (0.1μM) does not.

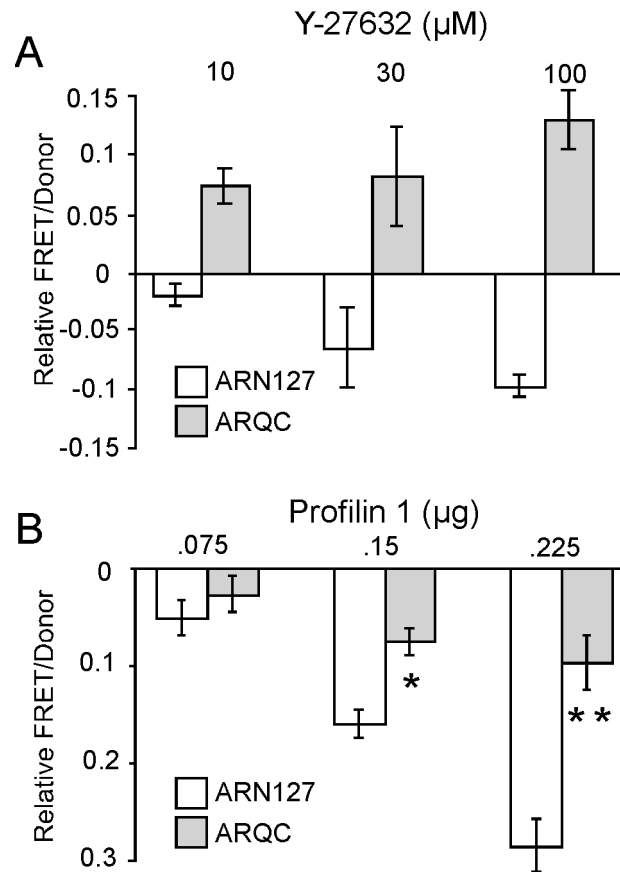
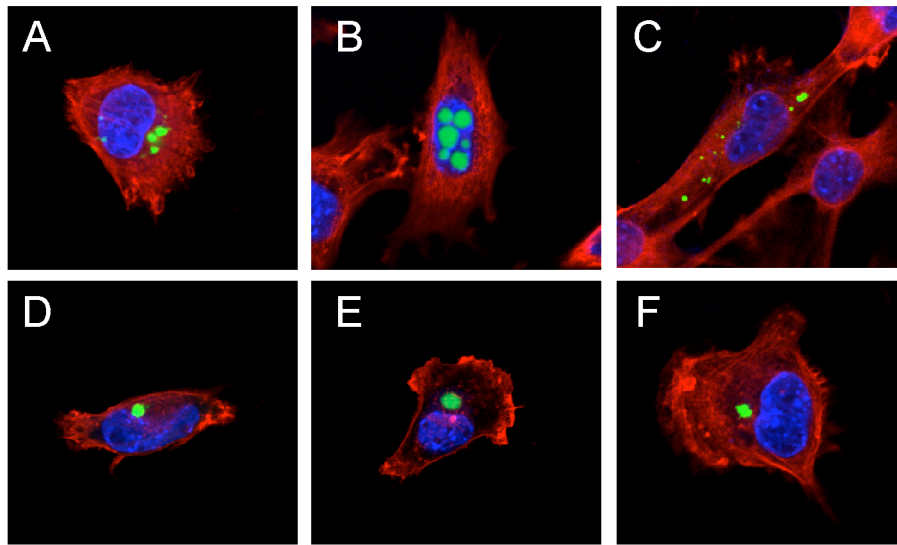


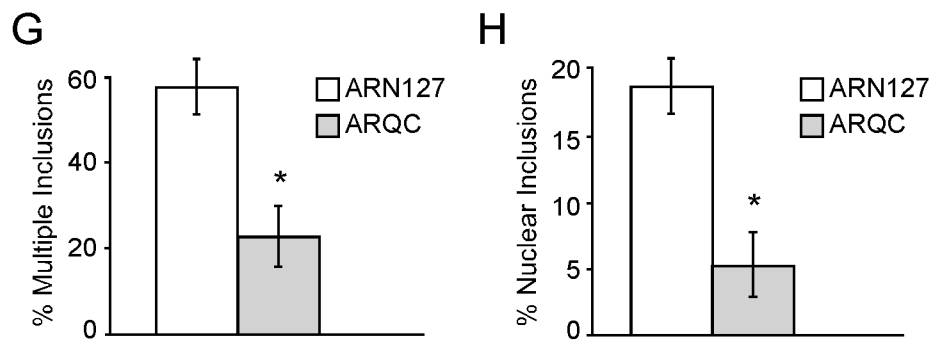
Figure 3: N50 of ARN127 mediates aggregation inhibition by actin- regulatory pathways.

A, Y-27632 inhibits aggregation of ARN127(65)-CFP/YFP and increases ARQC(65)-CFP/YFP aggregation. Cells expressing either ARN127 or ARQC fused to CFP/YFP were treated with 10, 30, or 100 μM Y-27632 for 24 hours. Relative FRET/donor measurements represent the change in aggregation from compounds compared to untreated cells. Y-27632 suppressed ARN127(65)-CFP/YFP aggregation, whereas it increased ARQC(65)-CFP/YFP aggregation. *B*, Profilin 1 decreases aggregation of ARN127(65)-CFP/YFP dose-dependently. Profilin 1 was cotransfected with polyglutamine proteins at increasing amounts, .075, .15, or .225 μg . This response was diminished for ARQC(65)-CFP/YFP. (*= $p < .01$, **= $p < .0001$, Student's t-test).

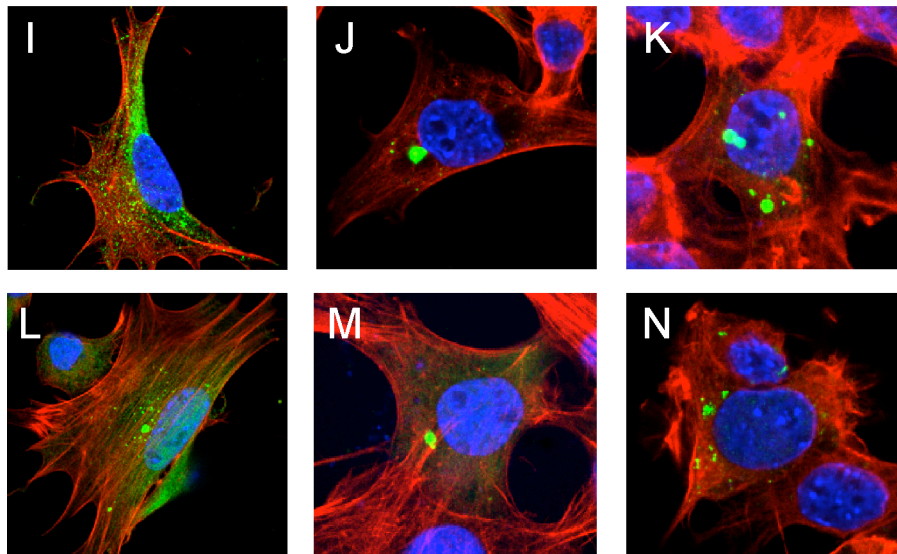
ARN127(65)-YFP



ARQC(65)-YFP



Htt exon 1 (97)-H4



HttQC(97)-H4

Figure 4: N50 of ARN127 influences inclusion type, number, and distribution.

A-C, Confocal images of ARN127(65)-YFP inclusions in C17.2 cells at 48h (60X). *D-F*, Confocal images of ARQC(65)-YFP inclusions in C17.2 cells at 48h (60X). YFP-tagged proteins are in green, F-actin is stained with rhodamine-phalloidin (red) and DNA is stained with DAPI (blue). *G*, ARN127(65)-YFP more often forms multiple inclusions per cell than ARQC(65)-YFP (*= $p < .002$, Student's t-test). *H*, ARN127(Q65)-YFP more often forms nuclear inclusions per cell than ARQC(65)-YFP (*= $p < .002$, Student's t-test). Averages are from three separate transfections, counting at least 100 cells each. *I-K*, Confocal images of Htt exon 1(97)-H4 in C17.2 cells at 48h (60X). *L-N*, Confocal images of HttQC(97)-H4 in C17.2 cells at 48h (60X). Immunofluorescence of HA-tagged Htt is in green, F-actin is stained with rhodamine-phalloidin (red) and DNA is stained with DAPI (blue). We did not observe significant differences in patterns of inclusion formation between the two Htt constructs.

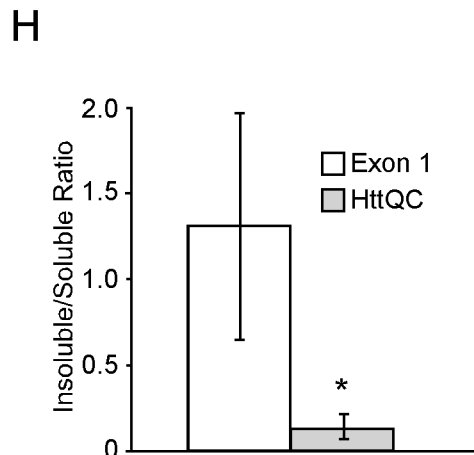
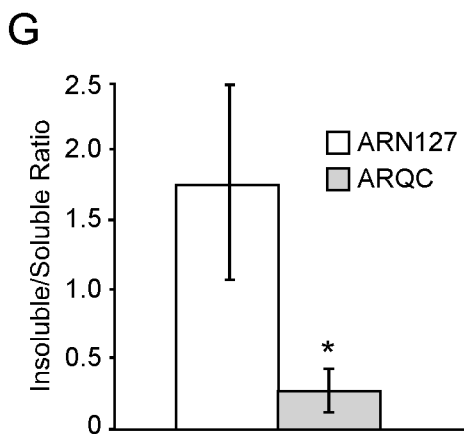
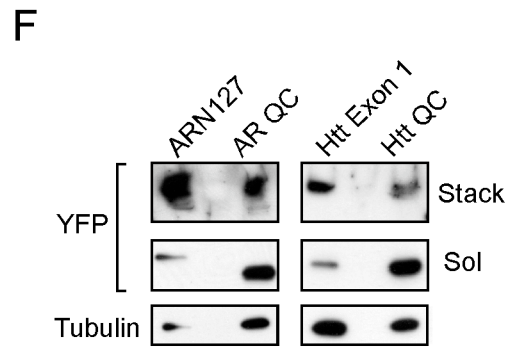
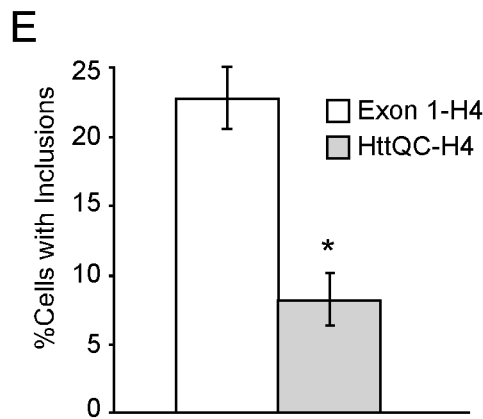
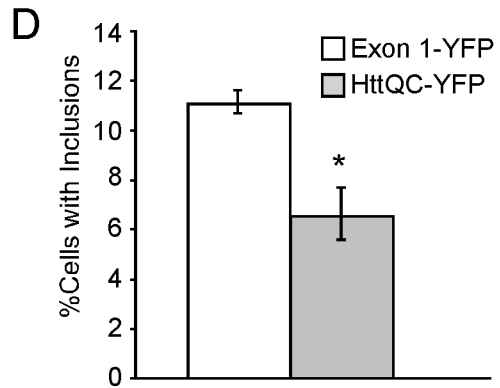
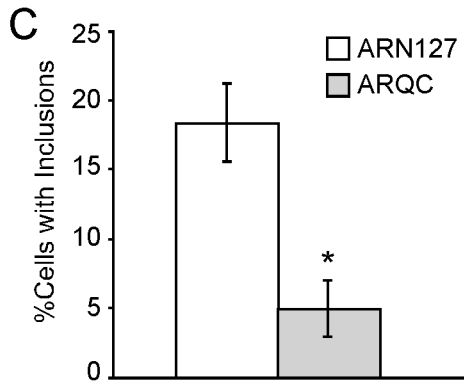
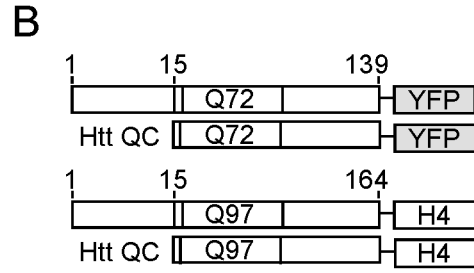
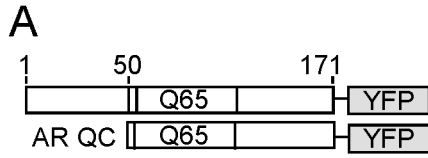


Figure 5: N50 of ARN127 and N14 of Htt exon 1 increase inclusion formation and detergent insolubility of aggregates.

HEK293 cells were transfected with the indicated constructs and evaluated after 24h. *A*, Schematic of AR fusion proteins, not to scale. *B*, Schematic of Htt fusion proteins, not to scale. The H4 sequence represents HIS-HA-HA-HIS epitopes. *C*, ARN127(65)-YFP forms more inclusions than ARQC(65)-YFP ($*=p<.0025$, Student's t-test). *D*, Htt exon 1(72)-YFP forms more inclusions than HttQC(72)-YFP ($*=p<.0025$, Student's t-test). *E*, Htt exon 1(97)-H4 forms more inclusions than HttQC(97)-H4 ($*=p<.005$, Student's t-test). *F*, ARN127(65)-YFP and Htt exon 1(72)-YFP form more SDS-insoluble aggregates than ARQC(65)-YFP and HttQC(72)-YFP. HEK293 cells were transiently transfected with the indicated constructs. After 24h, cells were lysed in 2% SDS sample buffer, and subjected to SDS-PAGE and western blot with YFP antibody. Stack indicates the SDS-insoluble higher molecular weight aggregates trapped in the stacking gel; Sol indicates the SDS-soluble monomers. Tubulin indicates loading control. Deletion of amino terminal peptides reduced the overall proportion of SDS-insoluble material detected in the stacking gel. *G*, Quantification of relative insoluble to soluble fractions of ARN127 vs. ARQC (n=3, $*=p<.05$, Student's t-test). *H*, Quantification of relative insoluble to soluble fractions of Htt exon 1 vs. HttQC (n=3, $*=p<.05$, Student's t-test). Quantification by Image J.

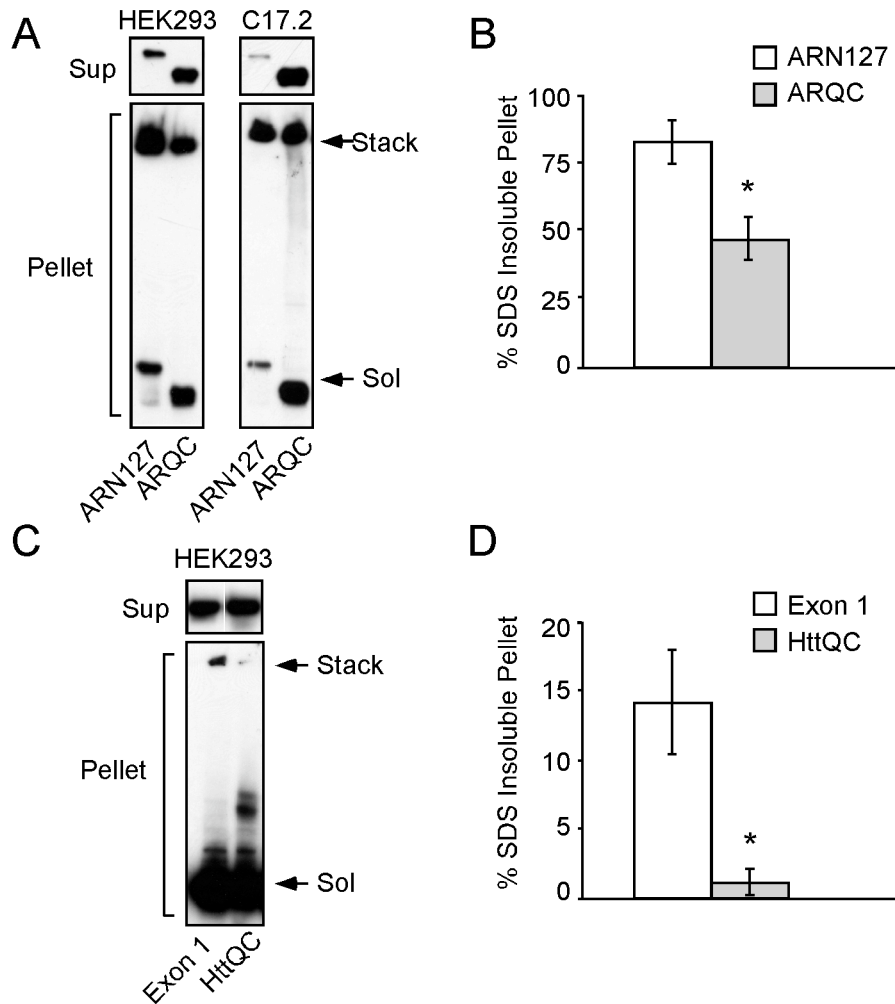
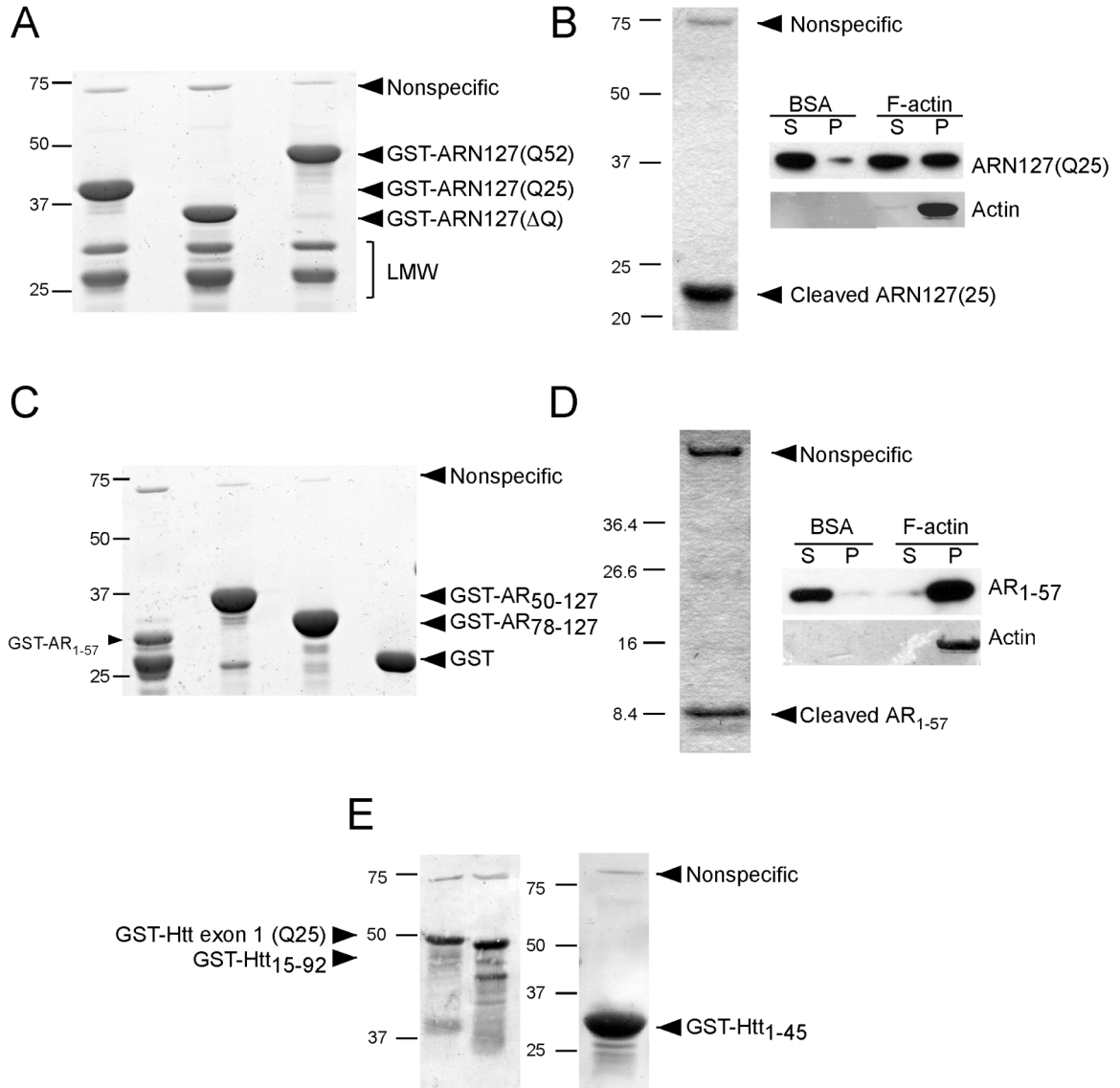


Figure 6: N50 of ARN127 and N14 of Htt exon 1 increase SDS-insoluble aggregate formation.

A, ARN127(65)-YFP forms more SDS-insoluble aggregates than ARQC(65)-YFP. HEK293 and C17.2 cells were transiently transfected with either ARN127(65)-YFP or ARQC(65)-YFP. After 24h, cells were syringe lysed and fractionated by centrifugation at 15,000 x g. Supernatant and pellet fractions were then resuspended in 2% SDS sample buffer and subjected to SDS-PAGE and western blot with YFP antibody. The soluble portions are shown in the upper panels, while the insoluble portions are shown in the lower panels. Stack indicates the SDS-insoluble higher molecular weight aggregates in the stacking gel; Sol indicates the SDS-soluble monomers. *B*, Quantification of SDS-insoluble aggregates for ARN127(65)-YFP and ARQC(65)-YFP from

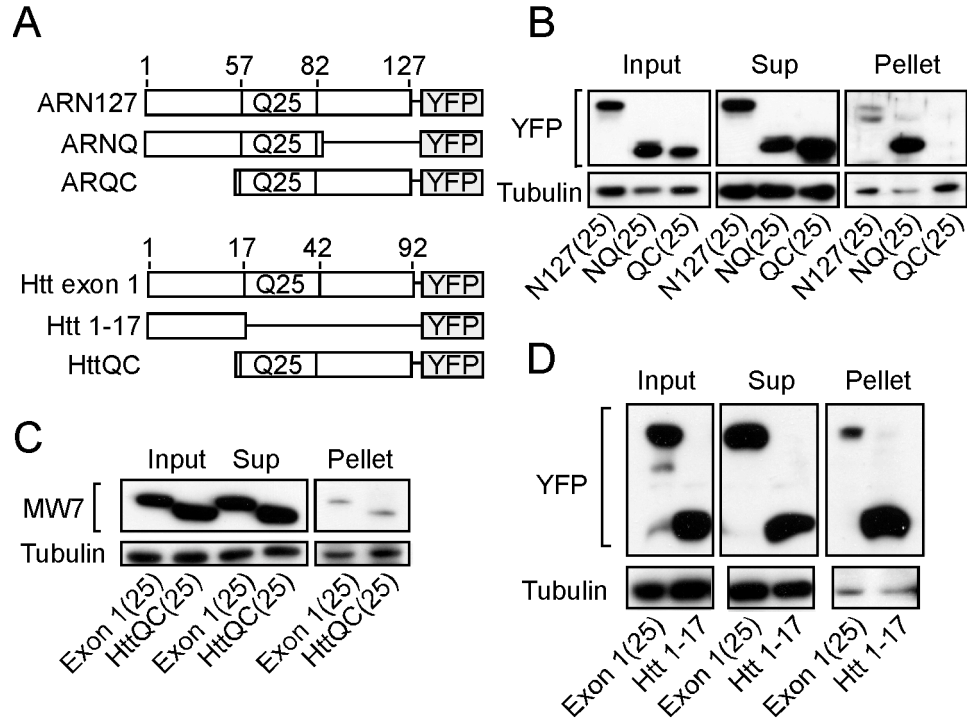
HEK293 cells after 24h. The insoluble fraction of ARN127(65)-YFP has significantly more SDS-insoluble material than ARQC(65)-YFP (n=3, $*=p<.001$, Student's t-test). *C*, Htt exon1(97)-H4 forms more insoluble aggregates than HttQC(97)-H4 in HEK293 cells after 24h. Cells were treated as above and subjected to SDS-PAGE and western blot with HA antibody. *D*, Quantification of SDS-insoluble aggregates for Htt exon1(97)-H4 and HttQC(97)-H4 from HEK293 cells after 24h. The insoluble fraction of Htt exon 1(97)-H4 has significantly more SDS-insoluble material than HttQC(97)-H4 (n=3, $*=p<.005$, Student's t-test).



Supplemental Figure 1: Purity of Recombinantly Purified Proteins

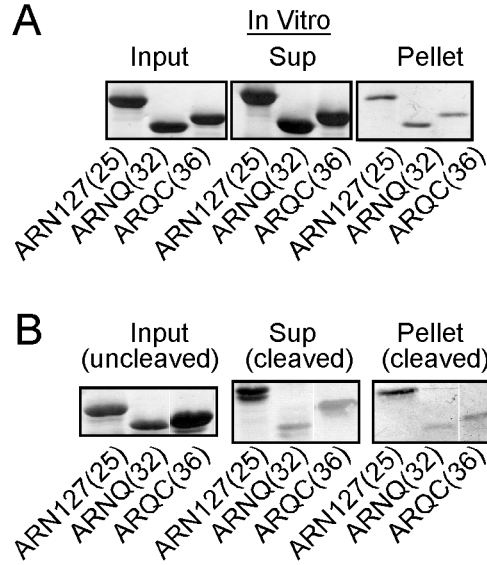
A, Coomassie stain of GST-ARN127(25), GST-ARN127(ΔQ), and GST-ARN127(52). Lower molecular weight (LMW) contaminants are indicated *B*, Cleaved ARN127(25) co-sediments with F-actin but is soluble in the absence of F-actin. GST-tagged ARN127(25) was treated with thrombin to cleave off the GST tag. 1μM of thrombin-cleaved ARN127(25) was mixed with 5μM F-actin as in Figure 1. Western blot was used to detect the N-terminus of AR (N-20 antibody) or Coomassie for actin. *C*, Coomassie stain of GST and GST-AR fragments, AR₁₋₅₇, AR₅₀₋₁₂₇, and AR₇₈₋₁₂₇, *D*, Cleaved AR₁₋₅₇ cosediments with F-actin but is soluble in the absence of

F-actin. GST-tagged AR₁₋₅₇ was treated with thrombin to cleave off the GST tag. 1 μM of thrombin-cleaved AR₁₋₅₇ was mixed with 5 μM F-actin. Western blot was used to detect the N-terminus of AR (N-20 antibody) or Coomassie stain for actin. *E*, Coomassie stain of GST-Htt exon 1(25), GST- Htt₁₋₄₅, and GST- Htt₁₅₋₉₂.



Supplemental Figure 2: N50 of ARN127 and N14 of Htt exon 1 mediate macromolecular interactions.

A, Schematic of AR and Htt peptides fused to YFP. *B*, The N-terminus of AR mediates macromolecular interactions. HEK293 cells were transfected with YFP fusion proteins, lysed in 1% Triton, and subjected to ultracentrifugation (100,000 x g). Supernatant and pellet fractions were analyzed via western blot with YFP antibody. ARN127(25)-YFP (top band) and ARNQ(25)-YFP are present in the Triton-insoluble pellets of HEK293 cells, while ARQC(25)-YFP is not. Tubulin indicates loading control. *C*, Deletion of the N14 region of Htt exon 1(25) does not affect Triton solubility. Supernatant and pellet fractions were analyzed via western blot with MW7 antibody (detects the C-terminus of Htt). Tubulin indicates loading control. *D*, The first 17aa of Htt alone fused to YFP cause it to become insoluble in cell lysates. Blots are probed with YFP. Tubulin indicates loading control.



Supplemental Figure 3: Inherent Solubility of GST peptides.

A, There are no differences in the inherent solubility of AR peptides. GST-tagged ARN127(25), ARNQ(32), and ARQC(36) are present in equal amounts in the pellet fractions after ultracentrifugation. *B*, Cleavage of AR peptides from GST does not unmask drastic differences in inherent solubility. GST was cleaved from ARN127(25), ARNQ(32), and ARQC(36) with thrombin protease at 4° C overnight. Peptides were ultracentrifuged (100,000 x g) and supernatant and pellet fractions were analyzed via SDS-PAGE and Coomassie stain.

Chapter 4

Investigation of F-actin Binding Motifs on Androgen Receptor and Huntingtin

Introduction

In Chapter 3, we showed that the N-terminus of both ARN127 and Htt exon1 directly bind to F-actin *in vitro*. We narrowed down the actin-binding domain to the first 50 amino acids of ARN127 (ARN50) and to the first 14 amino acids of Htt exon 1 (HttN14). Side-by-side comparison of the sequences show no apparent homology (Fig. 1). In this study, we went on to further investigate which exact binding-motifs are directly involved. We narrowed down the actin-binding domain of ARN127 to amino acids 20-50 via deletion analysis, performed circular dichroism (CD) to discern the secondary structure, and attempted to disrupt binding via site-directed mutagenesis. We investigated the N-terminus of Htt via microscopy and also attempted to find the exact binding motifs via point-mutagenesis.

Results

Amino acids 20-50 of ARN127 directly bind to F-actin in vitro.

To further narrow which domain of ARN50 may be involved in F-actin binding, we created 2 additional GST-tagged truncations containing either amino acids 1-20 (AR₁₋₂₀) or 20-57 (AR₂₀₋₅₇) (Fig. 2A) and performed the F-actin cosedimentation assay (Fig. 2B). We found that while AR₁₋₂₀ moderately associates with F-actin, AR₂₀₋₅₇ appears to bind almost completely (Fig. 2B), similar to ARN127 (Chapter 3, Fig. 2B). This data, in combination with previous mapping data (Chapter 3, Fig. 2), implicates amino acids 20-50 as necessary and sufficient for F-actin binding.

The N-terminus of Htt is a conserved, cytoplasmic domain.

Comparison of the first 17 amino acids of Htt (HttN17) reveals a striking conservation among vertebrate species (Fig. 3A), underscoring the importance of this region. By chance, analysis of the first 17 amino acids of another actin-binding protein found in yeast, termed Lifeact (Riedl et al., 2008), revealed striking sequence similarities (Fig 3A). When Lifeact is tagged with yellow-fluorescent protein (YFP), it can clearly be seen decorating actin-filaments (Fig. 3B). However, HttN17-YFP has a diffuse, predominantly perinuclear localization (Fig. 3C), in contrast to pure YFP, which is predominantly nuclear (Fig 3D). These data show that HttN17 is sufficient to drive YFP to the cytoplasm. The cytoplasmic nature of HttN17 has been described before and the perinuclear localization probably reflects interactions with the ER and Golgi (Atwal et al., 2007; Rockabrand et al., 2007). The lack of clear colocalization between HttN17-YFP and F-actin could reflect a transient interaction, or that the *in vitro* cosedimentation assay was not accurate. Further biochemical studies may be needed to discern this.

Point Mutagenesis of AR and Htt.

Htt exon 1 was recently crystallized and the first 17 amino acids were reported to form an α -helix (Kim et al., 2009), which is supported by previous biophysical findings (Atwal et al., 2007). Likewise, circular dichroism of ARN127 and AR₁₋₅₇ also indicated that the N-terminus is likely forming an α -helix (Fig. 4). To determine if the α -helix is essential for binding to F-actin *in vitro*, we introduced prolines to disrupt the helices. We replaced phenylalanine 23 in ARN127 (F23P) or methionine 8 in Htt exon 1 (M8P) and performed the F-actin cosedimentation assay. Amazingly, both the F23P and M8P mutants still bound to F-actin (Fig. 5A,B), indicating that disrupting the α -helix does not

disrupt the interaction. Since the first 17 amino acids of Htt represented a tractable motif to perform site-directed mutagenesis, we focused our remaining efforts on this domain. Actin is anionic and tends to interact with positively charged motifs (dos Remedios et al., 2003), so we next tested whether basic residues mediated the interaction in Htt exon 1. All lysines on the first 17 amino acids were mutated to alanines (K6A K9A K15A) and tested in the F-actin cosedimentation assay (Fig. 5B). However, these mutations were not sufficient to significantly abolish binding, indicating the interaction was not mediated via basic interactions. To determine if nonpolar or polar residues were required, various mutations were introduced on the third threonine and the fourth and seventh leucine (T3A L4S L7S) (Fig. 5). Again, disruption of these residues did not significantly affect binding. Comparison of HttN17 and Lifeact (Fig. 2A) reveal a strikingly similar phenylalanine, glutamic acid, serine (₁₁FES₁₃) motif. It remains to be tested if this motif is responsible for F-actin binding.

Discussion

In this chapter, we have further explored the actin-binding domains of ARN127 and Htt exon 1. We have found that amino acids 20-50 of ARN127 are necessary and sufficient for this interaction. Like Htt exon 1, the N-terminus of ARN127 appears to form an α -helix, though disrupting the helix does not abolish F-actin binding. AR₂₀₋₅₀ is rich in basic residues, which may be the next launching-point for mutagenic analysis. We have attempted to determine the exact motifs involved in Htt exon 1 binding, but to date have not found mutations that abolish this interaction *in vitro*. However, the ₁₁FES₁₃ motif, which also resides on Lifeact, is the next attractive candidate for mutagenesis.

Establishing F-actin point mutants for ARN127 and Htt exon 1 will be useful in directly testing the role of F-actin on both normal function and polyglutamine-induced dysfunction.

Material and Methods

Protein Purification. GST-ARN127 plasmids were grown in *E. Coli* Rosetta 2 (DE3) competent cells (Novagen). Protein expression was induced with 1mM (isopropyl β -D-thiogalactoside) IPTG for 3h at 37°C. GST-Htt plasmids were grown in *E. Coli* SURE competent cells (Stratagene). Protein expression was induced with 1mM IPTG for 3H at 30°C. Bacterial pellets were resuspended in resuspension buffer (PBS, .05% Tween, 1mM PMSF, protease inhibitor tablet (Roche)) and lysed by sonication and 1% Triton. GST-tagged proteins were precipitated with glutathione sepharose (Amersham Biosciences) and eluted with equal volumes of elution buffer (50mM Tris-HCl pH. 8m 10mM reduced glutathione). For cleavage of the GST-tag, 37.5 units of thrombin protease (Amersham Biosciences) was used for .5ml of GST-bound glutathione sepharose 4B (Amersham Sepharose) at 4°C overnight. Thrombin was removed from cleaved AR with benzamidine sepharose 6B (Amersham Biosciences). Protein concentration was quantified via Bradford assay and Coomassie staining via Image J.

F-actin Co-sedimentation. Non-muscle human actin (Cytoskeleton) was polymerized according to the manufacturer's instructions. 10 mg/ml of G-actin was polymerized with polymerization buffer (10X: 500mM KCl, 20mM MgCl₂, 10mM ATP) in general actin buffer (5mM Tris-HCl pH8.2mM CaCl₂, .5mM DTT, .2mM ATP) for 1h at RT. Equal

molar ratios of unlabeled phalloidin (Molecular Probes) were added to stabilize filaments. Recombinant, purified GST-tagged proteins were precleared via ultracentrifugation (100,000 x g for 30min at 4°C). Proteins were added to a pre-polymerized F-actin (4uM) or a BSA control for 1h on ice. Mixtures were ultracentrifuged for 30min at 100,000 x g at 4°C. Supernatants and pellets were subjected to SDS-PAGE. F-actin pellets were visualized via Coomassie stain. GST-proteins were probed via western blot using GST antibody (Santa Cruz Bioscience).

Site-Directed Mutagenesis.

All mutations were introduced followed the Qiagen Quikchange protocols. Briefly, complementary primers containing point mutations were designed and used modified PCR reaction. Methylated plasmid DNA was targeted with Dpn1. Plasmids with mutations were transformed in competent bacteria and clones were screened for mutations by sequencing.

Confocal Microscopy. All images were acquired on a C1sl confocal microscope (Nikon Instruments Inc.).

Immunofluorescence. NIH3T3 cells were mounted on polyornithine-coated glass coverslips 48 hours after transfection at a density of 20,000 cells per coverslip for 60X imaging. Cells were fixed in 4% paraformaldehyde, treated with .5% Triton, and blocked in 5% BSA for 1 hour. F-actin was visualized with rhodamine-conjugated phalloidin

(1:300, Molecular Probes) and the nucleus was stained with DAPI (Sigma). Coverslips were mounted with anti-fade mounting media (Invitrogen) and analyzed 24h later.

Circular Dichroism. 5 μ M recombinantly purified proteins cleaved and purified from GST tags as previously describe were used for CD analysis. Spectra were obtained from JASCO spectrometer.

AR ¹MEVQLGLGRVYPRPPSKTYRGAFQNLFQSVREVIQNPGRHPEAASAAPP⁵⁰

Htt ¹MATLEKLMKAFESLKSF¹⁷

FIGURE 1: Sequence Homology Comparison of AR₁₋₅₀ and Htt₁₋₁₇

The first 50 amino acids of AR or the first 17 amino acids of Htt are shown. They share no obvious sequence homology with each other. AR₁₋₅₀ does not appear to share homology with other known actin-binding motifs, while Htt₁₋₁₇ appears similar to the first 17 amino acids of another actin-binding protein, Lifeact.

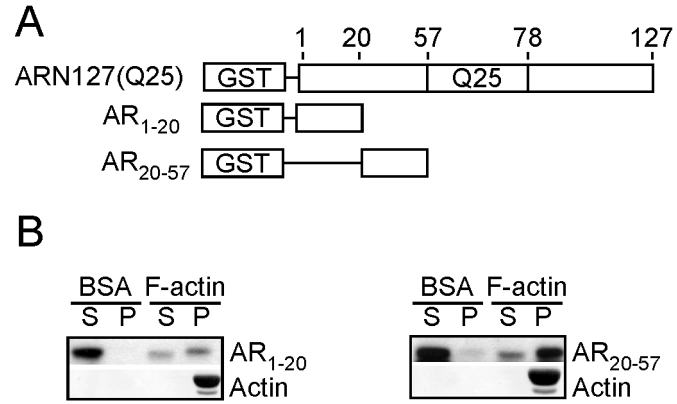


FIGURE 2: Amino Acids 20-57 are Necessary and Sufficient for Actin-Binding

A, Schematic of full-length ARN127, AR₁₋₂₀, and AR₂₀₋₅₇. *B*, AR₂₀₋₅₇ is necessary and sufficient for binding to F-actin via cosedimentation assay. AR₂₀₋₅₇ completely binds to F-actin, as does ARN127 (Chapter 3), while AR₁₋₂₀ only partially cosediments.

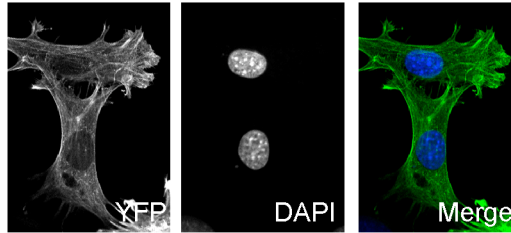
A

| | |
|-----------------------------------|-----------------------------------|
| Htt¹⁻¹⁷ | |
| <i>Homo sapien</i> | M A T L E K L M K A F E S L K S F |
| <i>Rattus norvegicus</i> | M A T L E K L M K A F E S L K S F |
| <i>Mus musculus</i> | M A T L E K L M K A F E S L K S F |
| <i>Sus scrofa</i> | M A T L E K L M K A F E S L K S F |
| <i>Bos taurus</i> | M A T L E K L M K A F E S L K S F |
| <i>Canis familiaris</i> | M A T L E K L M K A F E S L K S F |
| <i>Monodelphis domestica</i> | M A T L E K L M K A F E S L K S F |
| <i>Gallus gallus</i> | M A T M E K L M K A F E S L R S F |
| <i>Danio rerio</i> | M A T M E K L M K A F E S L K S F |
| <i>Tetraodon nigroviridis</i> | M A T M E K L M K A F E S L K S F |
| <i>Fugu rubripes</i> | M A T M E K L M K A F E S L K S F |
| Lifeact | |
| <i>Saccharomyces cerevisiae</i> | M G V A D L I K K F E S I S K E E |
| <i>Saccharomyces mikatae</i> | M G V A D L I K K F E S I S K E E |
| <i>Saccharomyces kudriavzevii</i> | M G V A D L I K K F E K I S N K K |
| <i>Saccharomyces bayanus</i> | M G V A D L I K K F E K F S K K E |
| <i>Saccharomyces castellii</i> | M G V A D L I Q K F E K Y A H V D |

Nonpolar
 Polar
 Acidic
 Basic
 Aromatic

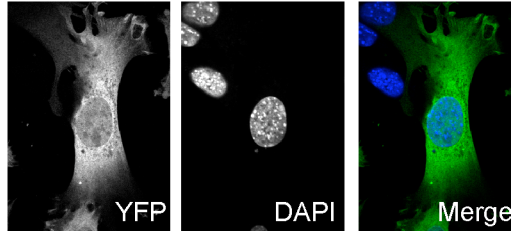
B

Lifeact-YFP



C

Htt¹⁻¹⁷-YFP



D

YFP

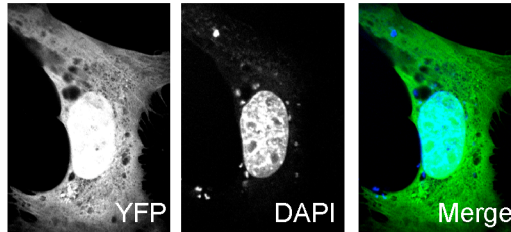


FIGURE 3: Htt₁₋₁₇ Sequence is Extremely Conserved among Vertebrates.

A, Evolutionary sequence homology comparison of Htt₁₋₁₇ and Lifeact. *B*, Lifeact-YFP decorates filamentous actin. DAPI stains the nucleus. *C*, Htt₁₋₁₇-YFP has a diffuse, cytoplasmic localization, in contrast to pure YFP, *D*, which has a predominantly nuclear localization.

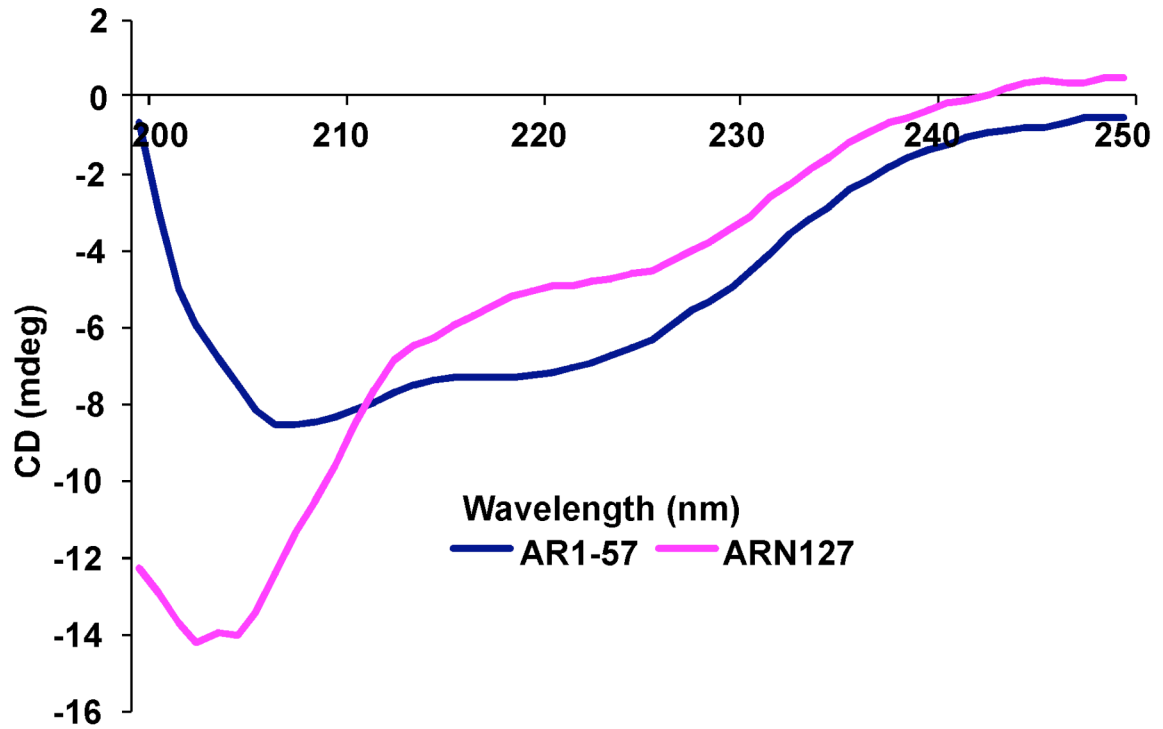


FIGURE 4: ARN127 and AR₁₋₅₇ have α -helical Composition.

Circular dichroism on recombinant ARN127 or AR₁₋₅₇ peptides reveal partial α -helical content for ARN127 (220nm) and mostly α -helical for AR₁₋₅₇.

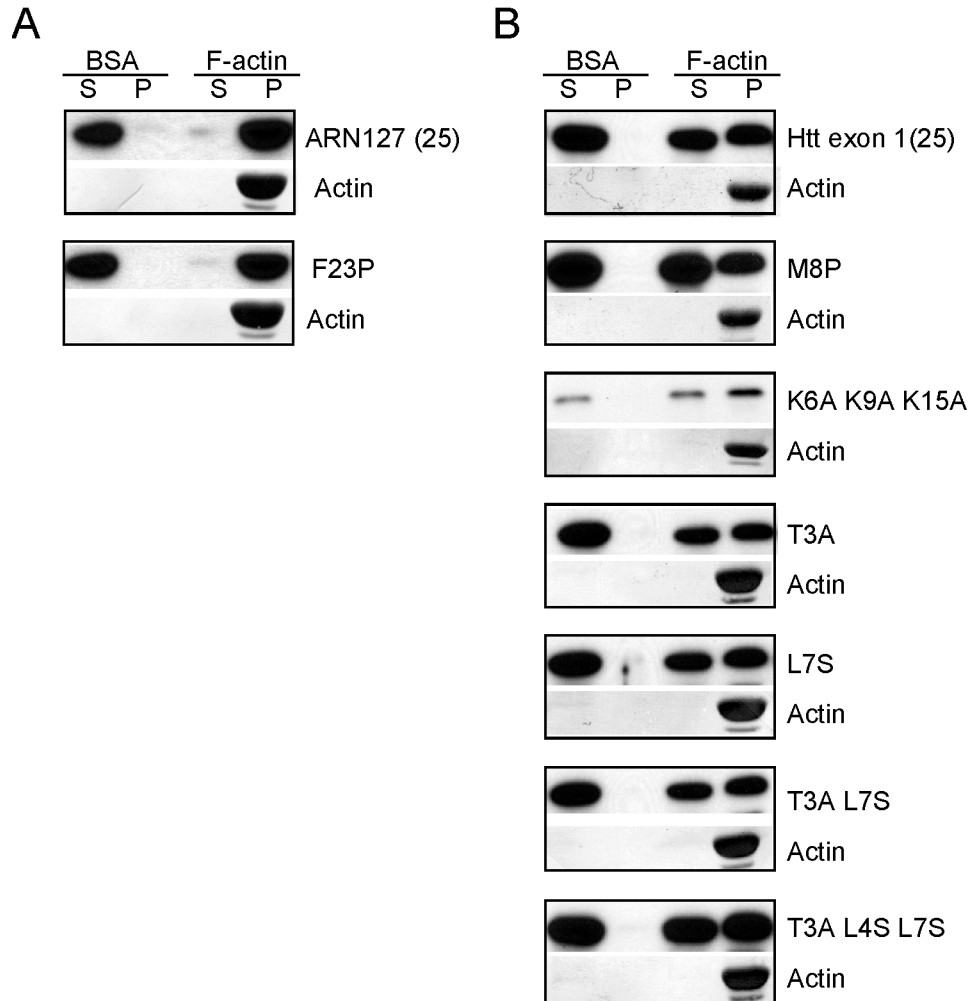


FIGURE 5: Site-Directed Mutagenesis of ARN127 (25) and Htt exon 1 (25).

A, Introducing a proline in the N-terminus of GST-ARN127 (25) does not abolish binding to F-actin via cosedimentation assay. F23P, phenylalanine to proline. *B*, Site-directed mutations were introduced to the N-terminus of GST-Htt exon 1 (25) and used in the F-actin cosedimentation assay. No mutations were sufficient to abolish F-actin binding. M8P, methionine to proline. K6/9/15A, lysine to alanine. T3A, threonine to alanine. L4/7S, leucine to serine.

Chapter 5
Conclusions and Future Directions

Conclusions and Future Directions

The discovery that FL-AR and FL-Htt interact with F-actin via phalloidin coprecipitation and F-actin cosedimentation assay (Chapter 2, Htt experiment originally performed by Jieya Shao) has opened up interesting implications for the normal function of these proteins. Both AR and Htt have clear precedents with actin-binding proteins (discussed in Chapter 2), and understanding how actin may interact normally with these polyglutamine proteins poses an interesting scientific question. Further, it raises the question if other polyglutamine proteins interact with actin and if there is a common role actin may play among all polyglutamine diseases. Remarkably, several other proteins involved in protein misfolding disorders, tau, α -synuclein, parkin, prion Sup35, have also been found to associate with actin (Esposito et al., 2007; Fulga et al., 2007; Ganusova et al., 2006; Huynh et al., 2000; Sousa et al., 2009). Given the abundance and importance of cellular actin, it is not all that surprising that many proteins bind to it, with no apparent conserved binding-sites. However, it will be interesting to determine if common binding partners contribute to the similar pathologies observed in protein misfolding diseases.

In Chapter 3, we investigated the role of N-terminal binding-domains on AR and Htt on polyglutamine aggregate formation. The discovery of Y-27632 as a potent modulator of polyglutamine aggregation sparked interest in actin as a potentially novel binding-partner. We found that the N-terminus of AR (ARN127) and Htt exon 1 directly bind to F-actin via an *in vitro* cosedimentation assay (Chapter 3). The first 50 amino acids of AR (Chapter 3), and more specifically, amino acids 20-50 (Chapter 4), mediate its interaction with F-actin, while the first 14 amino acids of Htt (Chapter 3) mediate its interaction. Amazingly, deletion of these domains has similar and dramatic

consequences on the intracellular polyglutamine aggregation of ARN127 and Htt exon 1. Both AR and Htt F-actin mutants form significantly fewer inclusions and become more SDS-soluble. ARN127 requires the N-terminal domain to be responsive to the anti-aggregation effects of Y-27632 and profilin. Furthermore, the N-terminal domain of AR regulates the distribution (nuclear vs. cytoplasmic) and number (multiple vs. single) of inclusions per cell. Likewise, the N-terminal domain promotes the formation of SDS-insoluble aggregates. Interestingly, while the inclusions of Htt and Htt mutant are visually similar, we see that like AR, the N-terminal domain promotes the formation of SDS-insoluble aggregates, indicating that the aggregates are biochemically distinct. Determining the differential toxicities of the aggregates, if any, and identifying protein interactions that can directly modulate toxicity will provide future therapeutic targets for these diseases.

Many factors can influence polyglutamine aggregation, including the biophysical properties of the protein, post-translational modifications, and protein-protein interactions. These factors are not mutually exclusive, and it can be conceived that biophysical properties may influence protein interaction. Conversely, protein interactions that change the conformation of sequences that flank the polyglutamine tract may in turn modulate the biophysical properties of the polyglutamine tract, thus potentially influencing a more or less toxic conformation (Kim et al., 2009). Without biophysical studies, we cannot predict how deletion of the F-actin binding domain influences its conformation and protein interactions. However, given that the actin-binding domains can regulate normal subcellular localization (Chapter 3), we can infer that protein-

interactions are very important for these domains and thus a potential modulator of polyglutamine aggregation.

As discussed in Chapter 4, we attempted to define the exact F-actin binding motifs for ARN127 and Htt exon 1. We found that amino acids 20-50 are necessary and sufficient for AR binding and future directions will involve introducing site-directed mutations to further identify the residues involved. We attempted to locate the exact motifs on Htt exon 1, but these have eluded us. Introducing prolines, disrupting basic, nonpolar, and polar residues has no effect on F-actin binding. By chance, we have noticed remarkable sequence similarity between HttN17 and Lifeact. Comparing the sequences reveals a common FES motif, and introducing point mutations in these residues is a current area of investigation. Identifying exact binding motifs will allow us to more precisely determine the role of F-actin binding on normal and aggregation-dependent cellular processes. It will also be important to better understand how the active modulation of actin dynamics (F-actin vs. G-actin) from proteins like profilin contribute to polyglutamine aggregation and if this is indeed based on a direct or indirect interaction.

It is now widely accepted that intermediate aggregate species, and not large end-stage polyglutamine inclusions, may be the culprit of polyglutamine toxicity (Arrasate et al., 2004; Li et al., 2007). We and others have now implicated protein interactions as important modulators of polyglutamine aggregation. Identifying protein interactions that targeting the toxic conformation of intermediate polyglutamine species and can ameliorate polyglutamine toxicity will thus be of great therapeutic value.

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