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SUMO Modification of Huntingtin and Huntington's Disease Pathology

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cohesins are released at metaphase by proteolytic cleavage (*22, 23*). Our finding that tankyrase 1 PARP activity is required to rescue the abnormal mitotic phenotype implicates a third posttranslational mechanism, poly(ADP-ribosyl)ation, in sister chromatid resolution. Whether telomeres are held together by cohesins or by telomere-specific proteins, such as TRF1 and its associated factors, remains to be determined.

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Supporting Online Material

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SUMO Modification of Huntingtin and Huntington's Disease Pathology

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Huntington's disease (HD) is characterized by the accumulation of a pathogenic protein, Huntingtin (Htt), that contains an abnormal polyglutamine expansion. Here, we report that a pathogenic fragment of Htt (Httex1p) can be modified either by small ubiquitin-like modifier (SUMO)–1 or by ubiquitin on identical lysine residues. In cultured cells, SUMOylation stabilizes Httex1p, reduces its ability to form aggregates, and promotes its capacity to repress transcription. In a *Drosophila* model of HD, SUMOylation of Httex1p exacerbates neurodegeneration, whereas ubiquitination of Httex1p abrogates neurodegeneration. Lysine mutations that prevent both SUMOylation and ubiquitination of Httex1p reduce HD pathology, indicating that the contribution of SUMOylation to HD pathology extends beyond preventing Htt ubiquitination and degradation.

HD is a dominant neurodegenerative disorder caused by the expansion of a polyglutamine [poly(Q)] repeat in Htt (*1*, *2*). In HD and other poly(Q) diseases, mutant proteins or pathogenic poly(Q) peptides produced by proteolytic processing aggregate into nuclear and/or cytosolic inclusions in neurons and in neuronal processes. These aggregates also contain other cellular proteins including transcription-regulating proteins, chaperones, proteasome subunits, and ubiquitin (*3–5*).

Pathogenic poly(Q) proteins can be modified in ways that change their cellular function or fate. Htt is subject to ubiquitination, which normally targets proteins for degradation (*6*, *7*). Mutations in ubiquitin ligases enhance poly(Q) toxicity in *Drosophila,* mouse, and cell models (*8–10*), whereas overexpression of Parkin, an E3 ubiquitin ligase, can reduce poly(Q) aggregation and suppress cytotoxicity (*11*). Thus, ubiquitination appears to reduce poly(Q) toxicity, presumably by promoting the degradation of toxic proteins.

SUMOylation—the covalent attachment of SUMO-1 to lysine residues—is a posttranslational modification process [for reviews, see (*12*, *13*)] that is biochemically similar to, but functionally distinct from, ubiquitination. SUMOylation can alter the function or subcellular localization of proteins, and competition between SUMO-1 and ubiquitin for identical target lysines can protect some proteins from degradation (*14–16*). The majority of SUMO-modified proteins are located in the nucleus (*17*), and SUMOylation can have a direct effect on nucleocytoplasmic transport. Here, we investigate how SUMOylation of Htt might affect HD pathogenesis.

Truncated Htt [Httex1p 97QP (Fig. 1A) (*18*)] and HIS–SUMO-1 colocalize when transfected into immortalized striatal nerve cells from cell line 12.7 (*19*) (Fig. 1B), reflecting either direct modification of Htt by SUMO-1 or colocalization of Htt with other SUMOylated proteins. To identify possible modifications of mutant Httex1p (*7*, *20*), which contains only three lysine residues [K^6, K^9, and K^{15} (Fig. 1A)], HIS–SUMO-1 or HIS-ubiquitin was coexpressed with Httex1p either intact or with the lysines mutated to arginine [$K^6 \rightarrow R^6$ (K6R), K9R, and K15R] in both striatal cells and HeLa cells. We also compared Htt fragments either with or without (97QP and 103Q, respectively) the proline-rich domain immediately following the poly(Q) region (Fig. 1A). Both proteins can be SUMOylated or ubiquitinated (Fig. 1C, lower panel) and a single primary SUMOylated species predominates, although more complex SUMO or ubiquitin modifications can be seen [Fig. 1D and supporting

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online material text] (*21*). The low levels of Htt detected in the HIS-ubiquitin–enriched fraction of cells transfected with the Htt triple lysine mutant K6R, K9R, and K15R (K6,9,15R) (Fig. 1E) may reflect ubiquitination of the N terminus of the protein (*22*). The proline motif enhances SUMO-1 modification but suppresses ubiquitination, consistent with a possible influence of other proteins that interact with this region.

All possible combinations of lysine mutants were generated within the first 17 amino acids of 97QP Httex1p, revealing that common residues are targeted for both SUMOylation and ubiquitination and specifically implicating residues K^6 and K^9 in these processes. When the lysine residues of Htt are mutated (double and triple lysine mutants), the abundance of Httex1p protein is reduced (Fig. 2A). Furthermore, the proline-rich region of Httex1p also appears to influence the abundance of soluble protein, given that Htt peptides with the prolines are more abundant than those without the prolines.

We explored three possible mechanisms whereby SUMO modification might influence pathology: aggregation, subcellular localization, and transcriptional dysregulation. To isolate the effects of SUMO modification, we fused SUMO to the N terminus of Htt (*23*) and compared the accumulation with and without the lysines and/or fusion with SUMO-1 (Fig. 2B). When fused to SUMO, the Htt peptide markedly accumulated, but it decreased when SUMOylation sites were eliminated.

Fusion of SUMO-1 to Httex1p also affects the aggregation properties of the protein. In a proliferating striatal neuronal cell line for which nuclear localization is minimal, expression of Httex1p 97QP leads to the formation of large Htt aggregates or inclusions (Fig. 2C, 97QP and 97QP K6,9,15R). Aggregate formation is also evident in the form of Htt-positive material that fails to penetrate the polyacrylamide gels of Western blots (Fig. 2B, 97QP and 97QP K6,9,15R). However, when "permanently" SUMOylated, the levels of disperse cytoplasmic protein are increased and inclusions are reduced (Fig. 2C, compare 97QP to SUMO-97QP and 97QP K6,9,15R to SUMO-97QP K6,9,15R). In addition, aggregates of both SUMO-97QP and SUMO-97QP K6,9,15R are absent from the stacking gels of Western blots and soluble levels are increased (Fig. 2B). Thus, surprisingly, in addition to stabilizing the protein, SUMOylation appears to reduce the formation of visible SDS-insoluble aggregates and increase disperse Htt staining in cells.

When the proline-rich domain is absent (103Q), expression of Httex1p does not produce visible inclusions or SDS-insoluble material (Fig. 2, B and D), although when 103Q is fused to green fluorescent protein (GFP), numerous inclusions are evident (*24*, *25*),

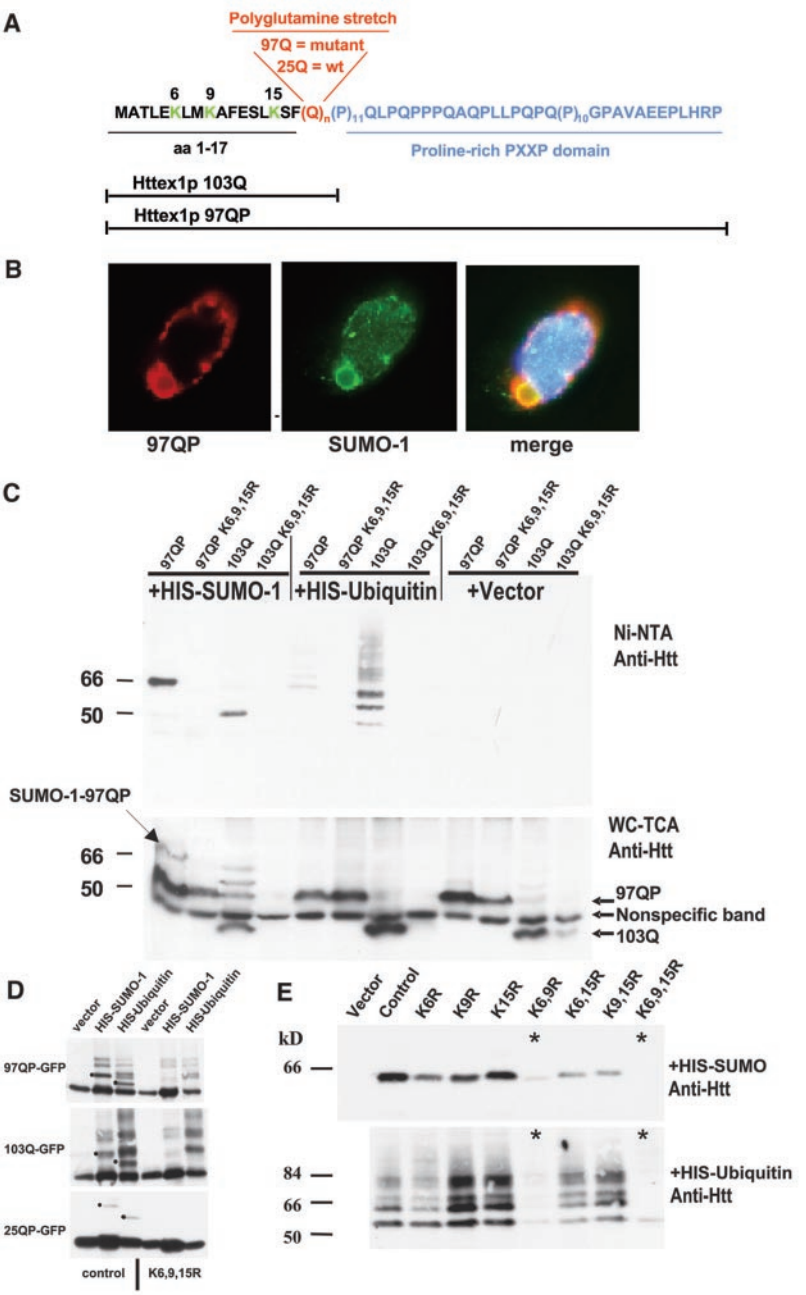

Fig. 1. Htt can be modified by SUMO-1 or ubiquitin and colocalizes with SUMO-1 in cell culture. (**A**) The sequence of the Httex1p fragment with the extent of the two transgene constructs indicated. wt, wild type; aa, amino acid; X, any amino acid. (**B**) SUMO-1 colocalizes with expanded Httex1p at the nuclear periphery and in inclusions of immortalized striatal neurons (cytoplasm not visible because of the fixation technique used). (**C**) Httex1p with and without the proline-rich region is modified by HIS–SUMO-1 or by HIS-ubiquitin in HeLa cells. Cells were cotranfected with plasmids expressing HIS-tagged SUMO-1 or ubiquitin, and Httex1p 97QP or 103Q. The tagged protein was enriched with Ni–nitrilotriacetic acid (NTA) magnetic nickel columns and detected by Western analysis with the antibody to Htt. Mutation of all lysine residues to arginine (K6,9,15R) inhibits both SUMOylation and ubiquitination of Httex1p. Of the initial Ni-NTA lysate, 10% was trichloroacetic acid (TCA) precipitated and subjected to Western analysis, showing relative levels of modified and unmodified Httex1p [whole-cell (WC)–TCA precipitates]. (**D**) Unexpanded 25QP Httex1p can be modified by SUMO-1 and ubiquitin. The 97QP-GFP, 103Q-GFP, and 25QP-GFP with wild-type lysines 6, 9, and 15 (control) or with these lysines mutated to arginine (K6,9,15R) were transiently cotransfected with a vector control, HIS–SUMO-1, or HIS-ubiquitin. Ni-NTA enrichment was performed as in (C). Unique SUMO-1 and ubiquitin-modified bands (black circles) are present in the control but not in the K6,9,15R mutants. (**E**) Mutation of the lysine residues singly and in combination significantly reduced or eliminated Httex1p modification by HIS–SUMO-1 or HIS-ubiquitin in HeLa cells, as demonstrated by Ni-NTA enrichment and Western analysis with antibodies to Htt.

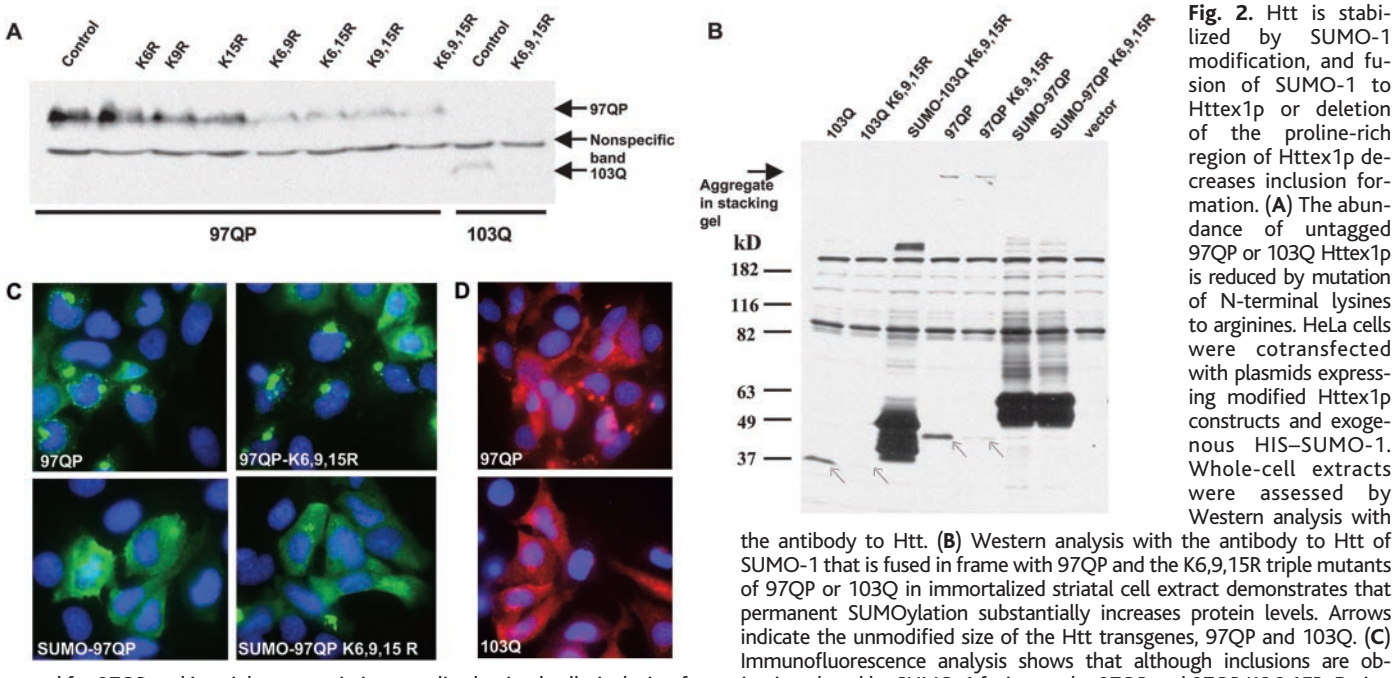

Fig. 2. Htt is stabilized by SUMO-1 modification, and fusion of SUMO-1 to Httex1p or deletion of the proline-rich region of Httex1p decreases inclusion formation. (**A**) The abundance of untagged 97QP or 103Q Httex1p is reduced by mutation of N-terminal lysines to arginines. HeLa cells were cotransfected with plasmids expressing modified Httex1p constructs and exogenous HIS–SUMO-1. Whole-cell extracts were assessed by Western analysis with the antibody to Htt. (**B**) Western analysis with the antibody to Htt of SUMO-1 that is fused in frame with 97QP and the K6,9,15R triple mutants of 97QP or 103Q in immortalized striatal cell extract demonstrates that permanent SUMOylation substantially increases protein levels. Arrows indicate the unmodified size of the Htt transgenes, 97QP and 103Q. (**C**) Immunofluorescence analysis shows that although inclusions are observed for 97QP and its triple mutant in immortalized striatal cells, inclusion formation is reduced by SUMO-1 fusion to the 97QP and 97QP K6,9,15R. Fusion of SUMO-1 to Htt 97QP produces diffuse staining predominantly in the cytoplasm in cells where expression is visible. (**D**) Immunofluorescence shows that inclusions are found in immortalized striatal cells expressing 97QP but not 103Q, demonstrating a role for the proline-rich region of Httex1p in aggregation.

indicating that inclusion formation is dependent on the presence of the proline-rich region (Fig. 2D). We considered whether the apparent SUMO-induced increase in Htt stability might simply reflect a redistribution of Htt from inclusions to soluble material. However, this is not the case because 103Q levels also increase when permanently SUMOylated, even though Q103 does not form inclusions (Fig. 2, B and D). We conclude that the proline-rich region of Httex1p is essential for inclusion formation and that SUMOylation of Httex1p both stabilizes the protein and reduces visible inclusion formation. It has been reported that ubiquitination of poly(Q) proteins can trigger the formation of visible protein aggregates (*26*). If ubiquitination is essential for inclusion formation, then blocking the putative ubiquitination sites by SUMOylation might alter the protein aggregation state as observed. In any case, the addition of SUMO appears to cause Htt to accumulate in a nonaggregated or early-aggregation state that is not visible by light microscopy. Recent studies have shown that soluble oligomers that precede aggregates are likely to be toxic (*27*), and SUMO modification of Htt may increase the levels of these potentially toxic oligomers.

Pathogenic processes in HD and other poly(Q) disorders appear to include repression of transcription by the mutant protein (*1*, *28*, *29*). Given that SUMO-1 has been shown to play a role in transcriptional regulation through modification of transcription factors (*30*), we asked whether SUMOylation of Htt can increase transcriptional repression. Because the fraction of Htt that is SUMOylated is quite low, we used permanently SUMOylated Htt to test the effects of modified Htt on transcriptional activity. We have previously shown that the WAF1-pGL3 and the Multidrug Resistance 1 (MDR1) gene promoters are repressed by expanded Htt (*31*). When transfected into striatal cells, SUMO-97QP appears to enhance the repression of the MDR1 promoter (Fig. 3). These observations demonstrate that SUMO modification of Htt can increase the suppressive effect of Htt on transcription.

How might SUMOylation affect transcription? SUMOylation could cause increased nuclear or subnuclear localization of SUMOylated Httex1p, given that SUMOylated proteins frequently localize to promyelocytic leukemia nuclear bodies that are implicated in transcriptional regulation (*32*). Modification of Htt by SUMO may increase the ability of Htt to be recruited to transcriptional repression complexes on chromatin. Alternatively, SUMOylation might alter cytoplasmic activity of Htt and either cause the release of a nuclear repressor that translocates to the nucleus (*33*) or cause the cytoplasmic retention of proteins that are necessary to activate transcription, such as cyclic adenosine monophosphate (cAMP) response element–binding protein (CREB)–binding protein (CBP).

Because SUMO-1 modification can influence nuclear localization of proteins (*17*) and SUMO modification occurs on the first 17 amino acids of Htt, we asked whether this region influences subcellular localization. We found that the first 17 amino acids of Htt can target proteins to the cytosol even when challenged with a strong nuclear localization sequence. This targeting may involve a previously unobserved CRM-1–independent export process or a cytoplasmic retention signal (fig. S1). In brain tissue from human HD patients, mouse models, and cell culture, mutant Htt protein is progressively localized from the cytoplasm to the nucleus (*1*, *10*, *29*). If nuclear localization of Htt is essential for HD pathogenesis, it is paradoxical that the pathogenic Htt fragment contains a cytoplasmic targeting signal. On the other hand, SUMO modification might preferentially mask this cytoplasmic retention signal in some cell types more than in others, allowing for different levels of nuclear localization and selective neuronal toxicity (*34*, *35*).

We next sought to genetically determine the relative contributions of SUMO and ubiquitin to pathogenesis. When mutant Httex1p (93QP) is expressed in all neurons of *Drosophila*, photoreceptor neurons are progressively lost and the integrity of the eye is compromised (*36*). However, neurodegeneration was greater in these flies than in siblings with lowered SUMOylation activity, which are heterozygous for a SUMO mutation, *smt3*/+ (Fig. 4A). In similar experiments, a reduction in ubiquitination activity modestly worsened pathology (Fig. 4B). Thus, SUMOylation makes pathology substantially worse, whereas ubiquitination makes pathology modestly better.

Because the same lysines are targeted by both SUMOylation and ubiquitination and

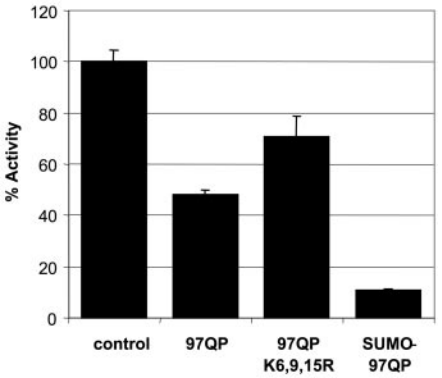

Fig. 3. SUMOylation of Htt can influence transcriptional repression. Luciferase assays were performed with immortalized striatal cells that were transiently cotransfected with the MDR1-luciferase reporter and with the pcDNA3.1 vector control, 97QP, 97QP K6,9,15R, or SUMO permanently fused to 97QP (SUMO-97QP). Error bars show mean + SEM.

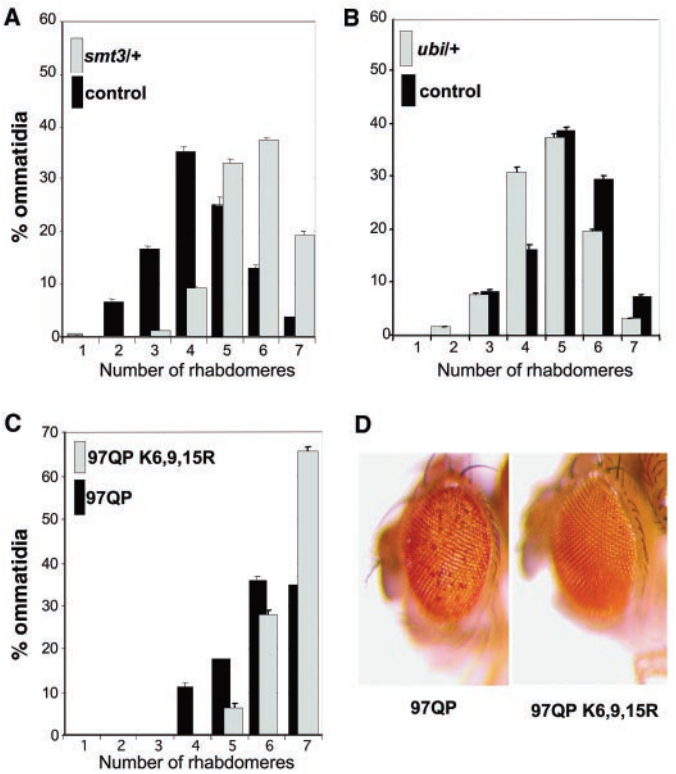

Fig. 4. Genetic reduction of SUMO activity in *Drosophila* reduces neurodegeneration in an HD fly model. (**A**) Neuropathology improves when SUMO levels are reduced. Flies expressing Httex1p Q93 ubiquitously in the nervous system under the control of the *elav*-GAL4 driver show extensive loss of photoreceptors (black bars). Normal flies show seven rhabdomeres in every ommatidium; the more extensive the degeneration, the fewer the number of rhabdomeres. When the level of SUMO activity is reduced by 50% in heterozygotes of the single SUMO gene in *Drosophila* (*smt3*/+), photoreceptor loss is substantially reduced (gray bars); thus, genetic reduction of SUMO activity rescues Htt Q93–mediated neuropathology. Student's *t* test: $P < 0.001$. (**B**) Neuropathology is only modestly increased when ubiquitin activity is reduced. When the level of ubiquitin activity is reduced by 50% in heterozygotes of the Ubi63E ubiquitin gene (*Ubi63E*/+), photoreceptor loss is modestly more severe (gray bars) than in controls expressing Htt Q93 in a normal background. Indeed, the increase in severity is barely significant statistically ($P < 0.060$). Thus, genetic reduction of ubiquitin activity only slightly exacerbates Htt Q93–mediated neuropathology. (**C**) Cytotoxicity is severely reduced by mutation of the three lysines in Htt 97QP. Transgenic *Drosophila* expressing Httex1p 97QP or Httex1p 97QP K6,9,15R under the control of *elav*-GAL4 at 27° were analyzed. Expression of the unmodified 97QP transgene produces significant photoreceptor loss (black bars). In contrast, when the transgene with the three lysines mutated is expressed, photoreceptor loss is substantially reduced ($P < 0.013$), indicating a strong attenuation of cytotoxicity by mutation of these lysines. Error bars in (A) to (C) show mean + SEM. (**D**) Transgenic *Drosophila* expressing Httex1p 97QP or Httex1p 97QP K6,9,15R under the control of *gmr*-GAL4 at 27° were analyzed. Expression of the unmodified Httex1p 97QP transgene produces a visible rough-eye phenotype with necrotic lesions indicative of cytotoxicity. In contrast, expression of the transgene with the three lysines mutated produces almost no detectable phenotype under the same conditions, again confirming a strong attenuation of cytotoxicity by mutation of these lysines.

because the global genetic reduction in both SUMO and ubiquitin can impact many cellular proteins and thereby indirectly affect the pathology of Htt, we sought to directly determine the influence of Htt modification on toxicity. Transgenic flies expressing Httex1p 97QP with or without the mutated lysines were compared. Mutation of the lysine residues substantially reduces pathology (Fig. 4C). Similar experiments using the *gmr*-GAL4 driver that expresses the transgenes in all cells of the *Drosophila* compound eye confirmed that the mutation of the lysine residues substantially suppresses cytotoxicity (Fig. 4D), indicating that the availability of these lysines is essential to the pathogenic process.

Is the role of SUMOylation simply to prevent ubiquitination? If mutating the lysines served only to reduce ubiquitination, then pathology should worsen (*9*, *10*, *37*, *38*). Instead, the opposite is true: Pathology is substantially reduced when the lysines are no longer available for posttranslational modification. These data indicate that the inability of Htt to be SUMOylated has a more notable impact on pathology than the reduced ability of Htt to be ubiquitinated and degraded.

Our findings demonstrate that Htt can be SUMOylated and suggest that SUMOylation can increase Htt accumulation, decrease aggregate formation (possibly increasing the amount of toxic oligomers), potentially mask a cytoplasmic retention signal, and promote nuclear repression of transcription. The impact of SUMOylation on HD pathogenesis in vivo is substantial. From a therapeutic standpoint, it is possible that decreasing expression of the SUMO-1 precursor, inhibiting SUMO-1 ligases, or increasing isopeptidase activity to remove SUMO-1 could reduce the level of SUMOylated Htt in neurons and suppress HD pathogenesis. The E3 ligase specific for attachment of SUMO-1 to Htt may present a particularly attractive therapeutic target.

The possibility that SUMO modification contributes to neurodegenerative disease has been suggested previously (*39*–*43*). An increase in SUMOylated proteins has been found in brain tissue from spinocerebellar ataxia type 1 patients and in transgenic mice (*43*). Furthermore, SUMO-1 colocalizes with expanded repeat Atrophin-1 in human brain tissue from dentatorubral-pallidoluysian atrophy patients (*42*). In two *Drosophila* models of poly(Q) disease, overexpression of a mutant form of the smt3-activating enzyme (UBA2) enhances neurodegeneration, implicating the SUMO conjugation pathway in poly(Q) pathogenesis (*39*). Recently, SUMO-2 was shown to reduce the production of the neurotoxic amyloid β peptide in cell culture (*40*, *44*), implicating components of the SUMOylation pathway in the onset or progression of Alzheimer's disease. These studies and ours suggest that SUMO modification may be an important modifier of the pathogenesis of HD and other neurodegenerative diseases.

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Materials and Methods
SOM Text
Fig. S1
References

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Development of a Human Adaptive Immune System in Cord Blood Cell–Transplanted Mice

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Because ethical restrictions limit in vivo studies of the human hemato-lymphoid system, substitute human to small animal xenotransplantation models have been employed. Existing models, however, sustain only limited development and maintenance of human lymphoid cells and rarely produce immune responses. Here we show that intrahepatic injection of CD34$^+$ human cord blood cells into conditioned newborn Rag2$^{-/-}\gamma_c^{-/-}$ mice leads to de novo development of B, T, and dendritic cells; formation of structured primary and secondary lymphoid organs; and production of functional immune responses. This provides a valuable model to study development and function of the human adaptive immune system in vivo.

Biomedical research in humans is restricted largely to in vitro assays that lack the components and complexity of a living organism. To overcome this limitation, substitute in vivo models have been developed in which human hematopoietic cells and tissues are transplanted into mice that are compromised in their capacity to reject xenogenic grafts. Engraftment was first reported after transfer of mature human peripheral blood leukocytes in severe combined immunodeficient mice (hu PBL-SCID mice) (*1*) and transplantation of blood-forming fetal liver cells, fetal bone, fetal thymus, and fetal lymph nodes in SCID mice (SCID-hu mice) (*2*, *3*). Subsequently, some level of human hematopoietic development was achieved by transplantation of blood-forming cells in NOD/SCID, NOD/SCIDβ2m$^{-/-}$, or NOD/SCID$\gamma_c^{-/-}$ mice (*4*–*7*). However, transfer of human cells in immunodeficient mice has, so far, not appeared to result in the de novo formation of a functional human adaptive immune system (*1*, *7*–*16*).

The liver contributes to perinatal hematopoiesis, and the hemato-lymphoid system expands most significantly during the first weeks of life. Thus, we reasoned that human hematopoietic stem and progenitor cells transplanted into the liver of immunodeficient newborn mice might find better conditions to engraft, expand, and reconstitute a human immune system. We transplanted newborn Rag2$^{-/-}\gamma_c^{-/-}$ mice, a mutant strain that lacks B, T, and NK cells (*17*, *18*), intrahepatically (i.h.) with CD34$^+$ cord blood cells (*19*). Mice were subsequently analyzed between weeks 4 and 26 of age, and human CD45$^+$ hematopoietic cells were detected in all animals (Fig. 1A). An increase in splenic and thymic cellularity was detectable, and all mice beyond 8 weeks developed mesenteric lymph nodes, several within size and cellularity of wild-type controls (Fig. 1B).

Most CD19$^+$ cells in bone marrow (BM) of engrafted mice were negative for surface immunoglobulin M (IgM) and CD20 expression, whereas spleen, lymph node, and blood CD19$^+$ cells expressed these antigens (fig. S1A). This was consistent with generation of B cells in BM and subsequent migration to spleen and lymph nodes. Human IgM was detectable in serum of young transplanted animals and increased in most over time; IgG was detected in older animals, demonstrating class switching of Ig isotypes (fig. S1B). Ig-producing cells were located in BM and spleen and correlated closely with numbers of CD19$^+$CD27$^+$CD138$^+$ plasma cells (fig. S1, C and D). Thus, in contrast to transplanted NOD/SCID mice where human B cells fail to produce Ig (*13*, *14*), full B cell maturation occurred in reconstituted Rag2$^{-/-}\gamma_c^{-/-}$ mice.

All thymi contained double-positive, as well as CD4 and CD8 single-positive, T

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SUMO Modification of Huntingtin and Huntington's Disease Pathology

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