Unfolded Protein Response Activation Reduces Secretion and Extracellular Aggregation of Amyloidogenic Immunoglobulin Light Chain

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Unfolded protein response activation reduces secretion and extracellular aggregation of amyloidogenic immunoglobulin light chain

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Light-chain amyloidosis (AL) is a degenerative disease characterized by the extracellular aggregation of a destabilized amyloidogenic Ig light chain (LC) secreted from a clonally expanded plasma cell. Current treatments for AL revolve around ablating the cancer plasma cell population using chemotherapy regimens. Unfortunately, this approach is limited to the ~70% of patients who do not exhibit significant organ proteotoxicity and can tolerate chemotherapy. Thus, identifying new therapeutic strategies to alleviate LC organ proteotoxicity should allow AL patients with significant cardiac and/or renal involvement to subsequently tolerate established chemotherapy treatments. Using a small-molecule screening approach, the unfolded protein response (UPR) was identified as a cellular signaling pathway whose activation selectively attenuates secretion of amyloidogenic LC, while not affecting secretion of a nonamyloidogenic LC. Activation of the UPR-associated transcription factors XBP1s and/or ATF6 in the absence of stress recapitulates the selective decrease in amyloidogenic LC secretion by remodeling the endoplasmic reticulum proteostasis network. Stress-independent activation of XBP1s, or especially ATF6, also attenuates extracellular aggregation of amyloidogenic LC into soluble aggregates. Collectively, our results show that stress-independent activation of these adaptive UPR transcription factors offers a therapeutic strategy to reduce proteotoxicity associated with LC aggregation.

ER proteostasis | amyloid

Light-chain amyloidosis (AL) afflicts 8–10 people per million per year, making it the most prominent systemic amyloid disease (1). AL is a gain-of-toxic function disease driven by a clonally expanded plasma cell that secretes amyloidogenic Ig light chains (LCs). These amyloidogenic LCs undergo extracellular misfolding and aggregation into proteotoxic soluble oligomers and amyloid fibrils that interact with distal tissues such as the kidney, heart, and gastrointestinal tract, leading to organ dysfunction and ultimately death by unknown proteotoxicity mechanism(s) (2).

The majority of AL patients must combat both a cancer (i.e., the clonally expanded plasma cell) and LC aggregation-associated proteotoxicity. The standard treatment for AL patients is chemotherapy (often combined with stem cell transplant) to eliminate the cancerous plasma cell population (3, 4). The proteasome inhibitor bortezomib, which takes advantage of the stress sensitivity of aggressively proliferating plasma cells, has transformed chemotherapy effectiveness (5–7). Regardless, ~30% of AL patients with substantial cardiac or renal LC proteotoxicity are too ill at diagnosis to tolerate chemotherapeutics (8–10). Thus, new strategies to reduce LC organ proteotoxicity must be developed to allow more AL patients to take advantage of chemotherapy.

LC aggregation requires conformational changes and a sufficient concentration of misfolded LC in plasma, which determine the rate and extent of its concentration-dependent aggregation. Over 500 distinct LC sequences, primarily of the lambda (λ) isotype (11), have been identified in AL amyloid deposits, reflecting the significant protein heterogeneity associated with AL proteotoxicity (12).

Amyloidogenic LCs generally contain an energetically destabilized variable domain that facilitates LC aggregation (2).

The amyloidogenic LC plasma concentration is determined by the extent of LC secretion from plasma cells and the rate of LC turnover in the serum. Secretion efficiency is largely dictated by the balance between folding vs. degradation, often referred to as “quality control,” in the endoplasmic reticulum (ER) (13). ER quality control is determined by the activity of the ER protein homeostasis (proteostasis) network comprising the protein folding, secretion, and degradation pathways (14–19). These pathways primarily function to prevent the secretion of destabilized, misfolding-prone proteins into the extracellular space (14–19).

Plasma cells have an evolutionarily enhanced ER and secretory pathway, enabling the proper folding and secretion of >1,000 IgGs per second (20, 21). Plasma cells normally secrete LC as IgGs [assemblies of two LCs and two heavy chains (HCs)], or as free LCs. However, in AL patients, LCs are predominantly secreted independent of HCs, sometimes as monomers, but generally as disulfide-linked homodimers called Bence–Jones proteins—the common precursor for LC aggregation (22, 23). Furthermore, LC ER quality

Significance

Light-chain amyloidosis (AL) is a devastating human disease involving the clonal expansion of a plasma cell and the secretion of destabilized, amyloidogenic immunoglobulin light chain (LCs). Secreted amyloidogenic LCs aggregate extracellularly, leading to proteotoxicity on distal tissues. Available therapeutic strategies to treat AL specifically target the cancerous plasma cell population. While this approach is effective in ~70% of patients, patients who present with substantial LC-related organ proteotoxicity are generally too sick to tolerate standard chemotherapeutics. Here, we show that stress-independent activation of unfolded protein response-associated transcription factors selectively reduces secretion of amyloidogenic LCs and decreases extracellular soluble LC aggregates associated with proteotoxicity in AL. These results identify a promising therapeutic strategy to treat AL patients unserved by current treatments.


The authors declare no conflict of interest.

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control is compromised in AL, allowing the efficient secretion of destabilized, amyloidogenic LC sequences that can aggregate extracellularly into proteotoxic soluble oligomers and amyloid fibrils.

Herein, we sought to identify signaling pathways that can be activated to reduce the secretion of destabilized, amyloidogenic LCs, thus reducing the extracellular LC concentration available for pathologic, concentration-dependent aggregation. Using a small-molecule screening approach, we identified that unfolded protein response (UPR) (24, 25) activation selectively reduces the secretion of a destabilized, amyloidogenic LC but not a more stable, nonamyloidogenic LC sequence. The UPR transcriptionally up-regulates ER quality control in response to ER stress, primarily through the transcription factors XBP1s and/or ATF6, comprising the adaptive UPR (25). Here, we show that stress-independent activation of XBP1s and especially ATF6 is sufficient to reduce amyloidogenic LC secretion and attenuate LC aggregation. Collectively, our results demonstrate that enhancing ER quality control via adaptive UPR activation represents a therapeutic strategy to ameliorate LC aggregation-associated proteotoxicity.

Results

Screening Reveals That UPR Activation Reduces Amyloidogenic LC Secretion. We developed a secretion reporter for LC by fusing the well-characterized AL-causing V7.6 LC containing both constant and variable LC domains (named ALLC) (26) to an enhanced Gaussia princeps luciferase (GLuc) reporter, ALLC-GLuc (Fig. L4) (27, 28). We used a similar GLuc fusion approach to quantify secretion of other proteins in a cell-based high-throughput screening (HTS) format (29). The secretion of ALLC-GLuc from HEK293-T Rex cells was observable by immunoblotting and luminescence (Fig. S1 A and B). ALLC secretion decreased upon inhibition of protein biosynthesis using cycloheximide (CHX) or after inhibition of global protein secretion using brefeldin A (Fig. SIC), as the secretion of GLuc, used as a control. ALLC-GLuc or GLuc was then stably incorporated into retinal pigment epithelium (ARPE-19) cells, as we previously optimized these professional secretory cells for HTS of GLuc fusion proteins such as ALLC-GLuc (29). The ALLC-GLuc secretion assay was miniaturized to a 384-well plate format, affording an HTS z-score (30) of 0.65 using cycloheximide as a control.

To identify biologic pathways that selectively reduce secretion of ALLC-GLuc, we screened the Library of Pharmacologically Active Compounds (LOPAC, Sigma Aldrich) in ARPE-19 cells secreting ALLC-GLuc or GLuc. The LOPAC was chosen because it is moderately sized (1,280 compounds), consists of compounds from major drug categories, and the biological targets for many small molecule components are known. We identified 15 molecules that significantly reduced ALLC-GLuc secretion (>25% reduction vs. vehicle control, 24-h treatment), while not affecting GLuc secretion (Fig. 1B, section shaded blue). Molecules that disrupted both ALLC-GLuc and GLuc secretion were not pursued further, as these likely reduce protein synthesis and/or disrupt global cell secretion (e.g., CHX and brefeldin A in Fig. SIC). Interestingly, the UPR activators thapsigargin (Tg), an inhibitor of sarcoplasmic reticulum Ca++-ATPase (31), and ellipticine (32) selectively reduced ALLC secretion relative to GLuc (Fig. 1B), suggesting that activation of the UPR could selectively influence LC secretion. The inability of a 24-h treatment with Tg to influence GLuc secretion is consistent with previous results (27).

To further examine the relationship between UPR activation and the reduction in ALLC-GLuc secretion, we performed an additional screen using HEK293-T Rex cells stably expressing firefly luciferase under the control of the ER stress-responsive element (ERSE) promoter (ERSE-FLuc, Fig. L4) (33). We found that 7 out of 15 molecules that selectively reduce ALLC-GLuc secretion by >25% also activated the ERSE-FLuc reporter >1.5 fold (Fig. 1C, blue), indicating that molecules that reduce ALLC-GLuc secretion are enriched for UPR activators.

Thapsigargin Selectively Reduces ALLC Secretion. To further understand the effect of ER stress-associated UPR activation on LC secretion, we used [35S]-metabolic labeling to measure the secretion of amyloidogenic ALLC fused to an N-terminal FLAG tag (F3 ALLC, Fig. 2A). We precultured cells in the absence or presence of Tg for 15 h to promote UPR-dependent remodeling of the ER proteostasis network before [35S]-metabolic labeling (Fig. 2A). F3ALLC was immunopurified from media and cell lysates at specific timepoints during a 4-h chase in nonradioactive media. These experiments show that Tg pretreatment reduces F3ALLC secretion by >40% (Fig. 2B). Importantly, because our Tg treatments do not drastically influence cell viability (Fig. S2 A and B), the Tg-dependent reduction in ALLC secretion cannot be attributed to cell toxicity. Furthermore the TG-dependent reduction in ALLC secretion is also observed in cells treated with the protein kinase R-like ER kinase (PERK) inhibitor GSK2606414 (34), demonstrating that the reduced secretion does not result from PERK-dependent translational attenuation (Fig. S2C).

No accumulation of F3 ALLC was observed in cell pellets following ER stress caused by Tg treatment (Fig. 2C), indicating that ALLC does not accumulate as intracellular aggregates. Reduced F3 ALLC secretion was associated with a decrease in total [35S]-labeled F3 ALLC (media + lysate) (Fig. 2D), suggesting that Tg-associated ER stress increases F3 ALLC degradation. Similar results were obtained with an untagged ALLC, confirming that the FLAG tag does not significantly influence ALLC secretion and/or degradation (Fig. S2 D and E). This Tg-dependent loss in total F3 ALLC cannot be reversed using proteasome inhibitors (bortezomib or MG132) or the p97 inhibitor ceyarestatin I [a potent inhibitor of ER-associated degradation (ERAD) (35)],
indicating that FTALLC is degraded following ER stress, at least in part through an ERAD-independent mechanism (Fig. 2E) (36). In contrast, the autophagy inhibitor chloroquine stabilized ALLC against Tg-induced degradation, indicating that Tg increases the autophagic degradation of ALLC (Fig. 2E).

Destabilized proteins are sensitive to UPR-dependent remodeling of the ER proteostasis network, whereas stable variants are generally less sensitive to this remodeling (37). Thus, we evaluated whether Tg-dependent reductions in LC secretion are also observed for a nonamyloidogenic, full-length V6 LC, FTV6 (38). Tg pretreatment did not reduce the secretion of [35S]-labeled, nonamyloidogenic FTJTO (Fig. 2F), indicating that Tg-dependent UPR activation selectively reduces the secretion of an amyloidogenic, but not a nonamyloidogenic LC.

LC amyloidogenicity is highly correlated with LC stability (38, 39). Thus, we compared the in vitro relative stability of recombinant proteins that have a folded, native β-sheet-rich Ig structure (Fig. S3 A and B). The relative stability of the ALLC and JTO LCs were compared by urea denaturation and monitored by tryptophan fluorescence (Fig. S3C). Although the ALLC unfolding transition is not completely reversible (Fig. S3A, black curve), the urea midpoint values for the denaturation curves (ALLC 1.8 M, JTO 2.6 M) indicate that ALLC is less stable than JTO (Fig. S3C). This suggests that the selective decrease in ALLC secretion afforded by Tg could be attributed to the inherent instability of the ALLC protein.

**XBP1s and/or ATF6 Activation Reduces the Secretion of Destabilized ALLC**

Previous work has indicated that remodeling of ER proteostasis pathways selectively influences the secretion of destabilized protein variants relative to more stable protein variants (13, 40, 41). Thus, Tg-dependent UPR activation could selectively decrease the secretion of destabilized ALLC through the transcriptional remodeling of the ER proteostasis network. To differentiate between the potential effects of Tg-induced ER stress and UPR-dependent remodeling of ER proteostasis pathways on ALLC secretion, we used a HEK293T-Rex-derived cell line that expresses both a ligand-regulatable ATF6 transcription factor [DHFR-ATF6; activated by the addition of the small molecule pharmacologic chaperone trimethoprim (TMP)] and a doxycycline (Dox)-inducible XBP1s transcription factor (cells referred to as HEK293T-DAX) (40). In these cells, the ATF6 or XBP1s transcriptional programs can be orthogonally controlled in the absence of ER stress, which enables analysis of the functional consequences of arm-selective UPR activation independently, or in combination (Fig. S4A) (40).

We treated HEK293T-DAX cells expressing FTALLC with Dox, TMP, or Tg and quantified the FTALLC levels in conditioned media by ELISA (Fig. 3A). Stress-independent activation of XBP1s or ATF6 reduced extracellular FTALLC levels to 68% and 48% of vehicle levels, respectively. Tg treatment lowered FTALLC levels to 34%. Reduced FTALLC secretion induced by XBP1s or ATF6 activation was dependent on transcription factor activity, as no reduction in FTALLC secretion was observed in TMP- or Dox-treated HEK293T-DAX cells—a control cell line stably expressing DHFR-YFP and Dox-inducible GFP (Fig. S4B) (40).

To probe the basis for the XBP1s- or ATF6-mediated reduction in ALLC secretion, we used [35S]-metabolic labeling (Fig. 3B–E). XBP1s, ATF6, or combinatorial activation lowered the relative secreted fraction of labeled ALLC by ∼40% following a 4-h chase (Fig. 3 B and C). In contrast, stress-independent activation of XBP1s and/or ATF6 did not affect the secretion of the nonamyloidogenic FTJTO (Fig. S4C). Notably, XBP1s- and ATF6-dependent reductions in ALLC secretion proceed through distinct mechanisms. XBP1s activation resulted in a 25% decrease in total [35S]-labeled FTALLC (Fig. 3D), as observed for Tg-treatment (Fig. 2D). This XBP1s-dependent decrease in total soluble ALLC was not
a result of intracellular accumulation of FT-ALLC in the cell pellet (Fig. S4D), implying the loss of FT-ALLC is due to degradation and not intracellular aggregation. ATF6 activation, in contrast, did not decrease total ALLC recovery in our [35S] metabolic labeling experiments (Fig. 3D) but instead resulted in an increase in intracellular [35S]-labeled FT-ALLC (Fig. S2E). Coactivation of XBP1s and ATF6 decreases FT-ALLC recovery to that observed for XBP1s activation. Similar results were observed with untagged ALLC (Fig. S5).

Altered processing of ALLC in the ER after XBP1s and/or ATF6 activation likely reflects distinct interactions between ALLC and ER proteostasis network components differentially induced by these transcriptional programs. We used an in situ cross-linking and immunopurification approach to sensitively measure the intracellular associations between ALLC and ER chaperones such as BiP (GRP78) and GRP94 (Fig. S6A)—two ER chaperones known to interact with LC (42, 43). As predicted, ATF6 activation, but not XBP1s activation, resulted in a significant increase in the association between ALLC and the ATF6-regulated ER chaperones BiP and GRP94 (Fig. 3 F and G), reflecting the increased levels of these two ER chaperones afforded by ATF6 activation (40). The increased association between ALLC and these ER proteostasis factors provides a mechanism to explain the ATF6-induced intracellular retention of destabilized, aggregation-prone ALLC in the ER lumen. ATF6 activation also increased interactions between FT-JTO and BiP and GRP94 (Fig. S6B), albeit to a lesser extent than that observed for ALLC (Fig. 3 F and G).

XBP1s and/or ATF6 Decreases Extracellular Aggregation of Secreted ALLC. Lowering the secretion of an amyloidogenic LC should directly ameliorate AL organ proteotoxicity by reducing the extra- cellular concentration and therefore the extracellular aggregation of amyloidogenic LCs. To scrutinize this hypothesis, we examined the concentration-dependent aggregation of recombinant ALLC by monitoring turbidity of samples heated to 44 °C (Fig. 4 A and B). Reducing the concentration of ALLC significantly reduced the extent (Fig. 4A) and rate of aggregation (Fig. 4B), demonstrating that recombinant ALLC aggregates through a concentration-dependent mechanism.

Because XBP1s and/or ATF6 activation decreases the secretion of amyloidogenic LC, we reasoned that the stress-independent activation of these transcriptional programs would similarly decrease extracellular ALLC aggregation. Heating the conditioned media of cells expressing FT-ALLC to 55 °C for 0–24 h produced large soluble aggregates that increase with time, as discerned by blue native polyacrylamide gel electrophoresis (BN-PAGE) (Fig. S7A) and gel filtration chromatography (Fig. S7B). Reducing the extracellular concentration of FT-ALLC in conditioned media by dilution with media conditioned on GFP-transfected cells shows that cell-secreted ALLC aggregation is concentration dependent (Fig. 4C), where a 1:1 [50% (vol/vol)] dilution of ALLC conditioned media nearly eliminates ALLC aggregates. We next evaluated whether stress-independent XBP1s and/or ATF6 activation similarly attenuates ALLC aggregation. We collected conditioned media from FT-ALLC-expressing HEK293ΔXAS cells following XBP1s and/or ATF6 activation and measured ALLC aggregation using BN-PAGE. ATF6 activation resulted in a 73% loss of soluble FT-ALLC aggregates (55 °C, 8 h incubation; Fig. 4 D and E), whereas XBP1s activation resulted in a 20% loss, and activating both XBP1s and ATF6 reduced aggregate formation by 60% (Fig. 4 D and E). As expected, the reduction in ALLC aggregation corresponds with a decrease in total extracellular ALLC (Fig. 4 D and E). Interestingly, the reduction in ALLC
aggregation was more pronounced than expected from just the reduction in total extracellular ALLC, suggesting that XBPIs and/or ATF6 activation could also influence ALLC aggregation through other mechanisms. Importantly, we do not observe significant associations between ALLC and the ER chaperones BiP and GRP94 in conditioned media, indicating that the reduction in extracellular ALLC aggregation cannot be attributed to increased association with these ER chaperones secreted from cells (Fig. S84). ALLC secretion and aggregation was not sensitive to treatment with Dox, TMP, or both in the control HEK293ΔV cells (Fig. S88), demonstrating that the reduced aggregation of ALLC observed in HEK293ΔV requires XBPIs and/or ATF6 transcriptional activity.

Discussion

We show that stress-independent activation of one or both of the adaptive UPR-associated transcription factors XBPIs or especially ATF6 reduces the secretion and extracellular concentration of amyloidogenic ALLC, reducing soluble ALLC aggregate levels. This strategy has the potential to be clinically meaningful, as a >50% reduction in the amount of circulating amyloidogenic LC mediated by chemotherapeutic methods correlates with a substantial survival benefit (44). Lowering the amount of amyloidogenic LC secreted from plasma cells into the blood by UPR activation should be useful in patients with significant cardiac and/or renal involvement. This approach, with time, should dramatically reduce amyloidogenic LC oligomer levels and thus proteotoxicity, enabling the patient to tolerate established chemotherapy regimens. Arm-selective UPR activation could also be used in combination with chemotherapy approaches to further reduce the amount of circulating LC and organ proteotoxicity. Lastly, the approach could potentially be useful in cases of AL recurrence, decreasing the amyloidogenic LC secretion and organ proteotoxicity in chemotherapy-resistant AL patients.

Partitioning of amyloidogenic LCs between folding and trafficking vs. degradation pathways is determined by the interactions between the destabilized LC and ER proteostasis network components. The stoichiometry of the components of the ER proteostasis network is regulated by the activated signaling arms of the UPR (40). This in turn determines the specific ER proteostasis factors that interact with amyloidogenic LC and, thus, dictates the partitioning of LC between retention, secretion, or degradation in the ER lumen. The degradation of FVALLC upon XBPIs activation accounts for most of the observed reduction in secretion. In contrast, ATF6 activation does not induce ALLC degradation in HEK293ΔV but instead leads to intracellular retention of ALLC, consistent with the increased interactions between ALLC and the ER chaperones BiP and GRP94. In AL-patient plasma cells, the retention of an amyloidogenic LC could sensitize the cells to death by subsequent chemotherapeutic strategies, or by itself be cytotoxic. Alternatively, degradation could eventually occur with repeated but periodic ATF6 activation, although this potential mechanism needs to be further explored. Increasing ER quality control by arm-selective UPR activation appears to be a general approach to reduce the secretion of destabilized, aggregation-prone proteins (37). Activation of ATF6 selectively reduces the secretion of destabilized, amyloidogenic transthyretin mutants from hepatocytes and can reduce intracellular accumulation of mutant rhodopsin, while not affecting secretion of the wild-type proteins (40, 41), providing strong support for this hypothesis.

Based on the results described above, we are now seeking small molecules that activate the ATF6 and/or XBPIs arms of the UPR. These small molecules would transcriptionally remodel the ER proteostasis network in plasma cells, reducing the secretion of energetically destabilized LCs irrespective of their primary structure, obviating the need for sequence- and conformation-specific AL drug design challenged by the significant sequence heterogeneity associated with LC proteotoxicity in AL. Furthermore, based on other reports demonstrating the capacity of XBPIs and/or ATF6 activation to influence aberrant ER quality control linked to disease-associated proteotoxicity (40, 41), establishing small molecules that increase ER quality control is expected to have significant potential for the treatment of additional protein aggregation diseases.

Materials and Methods

Plasmids, Cell Culture, HTS, and Recombinant Protein Production. Detailed protocols can be found in SI Materials and Methods.

Pulse-Chase Experiments. HEK293ΔV or HEK293T-Rex cells plated on poly-lysine coated plates were metabolically labeled in DMEM –Cys/-Met (CellGro) supplemented with glutamine, penicillin/streptomycin, 10% dialyzed FBS, and [35S]-Translabel (MP Biomedical) for 30 min. Cells were washed with complete media and incubated in DMEM for the indicated times and harvested at the indicated times. In the case of added inhibitors, cells were pretreated for 4 h at the indicated concentrations, and inhibitors were added to the pulse and chase media. lysates were prepared in radioimmunoprecipitation assay buffer plus protease inhibitor mixture (Roche) with 10 mM GdCl3. Proteins were immunopurified using anti-FLAG M1 agarose beads (Sigma). Protein was eluted by boiling in Laemmli buffer + 100 mM DTT, and samples were separated by SDS-PAGE. The gels were dried, exposed to phosphorimager plates (GE Healthcare),
and imaged with a Typhoon imager. Band intensities were quantified in ImageQuant. Fraction secreted was calculated using the equation: Fraction secreted = [extracellular \( [^{35}S] \)-LC signal at t = 0] / [extracellular \( [^{35}S] \)-LC signal at t = 0 + intracellular \( [^{35}S] \)-LC signal at t = 0]. Fraction remaining was calculated using the equation: [intracellular \( [^{35}S] \)-LC signal at t = 0 + intracellular \( [^{35}S] \)-LC signal at t = 0] / [extracellular \( [^{35}S] \)-LC signal at t = 0].

**Conditioned Media Aggregation and Blue Native PAGE.** HEK293\(^{2AX}\) cells expressing LC or GFP control were treated with vehicle, doxycycline (Doxx), trimethoprim (TMP), or the combination for 16 h. The medium was removed, centrifuged at 200 \( \times \) g to remove cell debris, transferred to a new tube, and probe inhibitor mixture (Roche) added. The medium was incubated at 55 °C with aliquots removed at the indicated time points.

Conditioned medium was added to blue native PAGE loading dye (10% glycerol, 0.5% Coomassie G-250) and then loaded onto 3

\[ \text{gradient gels (invitrogen)} \]. The cathode buffer contained 50 mM Tricine and 15 mM Bis-Tris, pH 7.0 with 0.02% Cosmacise G-250. The anode buffer contained 50 mM Bis-Tris pH 7.0. The gels were transferred onto PVDF membranes, and LC was detected by polyclonal anti-human lambda LC (Bethyl Laboratories), followed by HRP-conjugated secondary antibodies. The blots were imaged using a chemiluminescence substrate (Luminata Forte Western Luminescence Substrate, Millipore) and imaged by film or with a Bio-Rad scanner.

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