Glucocorticoid elevation of dexamethasone-induced gene 2 (Dig2/RTP801/REDD1) mediates autophagy in lymphocytes

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Glucocorticoid hormones, including dexamethasone, induce apoptosis in lymphocytes and consequently are used clinically as chemotherapeutic agents in many hematologic malignancies. Dexamethasone also induces autophagy in lymphocytes, although the mechanism is not fully elucidated. Through gene expression analysis we found that dexamethasone induces the expression of a gene encoding a stress response protein variously referred to as Dig2, RTP801 or REDD1. This protein is reported to inhibit mTOR signaling. Since autophagy is one outcome of mTOR inhibition, we investigated the hypothesis that Dig2/RTP801/REDD1 elevation contributes to autophagy induction in dexamethasone-treated lymphocytes. In support of this hypothesis, RNAi-mediated suppression of Dig2/RTP801/REDD1 reduces mTOR inhibition and autophagy in glucocorticoid-treated lymphocytes. We observed similar results in Dig2/RTP801/REDD1 knockout murine thymocytes treated with dexamethasone. Dig2/RTP801/REDD1 knockdown also leads to increased levels of dexamethasone-induced cell death, suggesting Dig2/RTP801/REDD1-mediated autophagy promotes cell survival. Collectively, these findings demonstrate for the first time that elevation of Dig2/RTP801/REDD1 contributes to the induction of autophagy.

The ability of glucocorticosteroid hormones (i.e., glucocorticoids, adrenal corticosteroids) to induce atrophy of the thymus gland and other lymphoid organs was recognized in the first half of the twentieth century (1). This observation was of profound importance, as it engendered the use of glucocorticoids both as anti-inflammatory and immunosuppressive agents (2) and as therapeutic agents for lymphoid malignancies (3). Today, synthetic glucocorticoids (e.g., prednisone, dexamethasone) are among the most effective anti-inflammatory and immunosuppressant agents employed in clinical medicine (4). Also, these same synthetic glucocorticoids continue to play a critical role in the treatment of lymphoid malignancies (5,6). Because of their therapeutic importance, understanding the fundamental mechanism(s) by which glucocorticoids regulate lymphocyte function and viability is of considerable importance.

Two landmark discoveries served to focus research in this area for several decades. First, Tompkins and coworkers provided considerable insight into the molecular mechanism of glucocorticoid-induced cell death by proving that it is mediated by the glucocorticoid receptor, a ligand-regulated transcription factor (7-9). Second, the discovery that glucocorticoids kill thymocytes by inducing apoptosis (10) directed research in this area squarely toward understanding the mechanism of glucocorticoid-induced apoptosis by identifying glucocorticoid-regulated "death genes." To this end, a number of laboratories have used gene expression profiling in a quest to identify putative "glucocorticoid-induced death gene(s)". These studies, carried out in a variety of lymphoma/leukemia cell lines and primary leukemia cells, identified a vast array of genes regulated by the synthetic glucocorticoids prednisone and dexamethasone (11-19). Of all the glucocorticoid-induced genes identified through this experimental strategy, one of the genes most directly related to apoptosis induction is that encoding the proapoptotic protein Bim (12,20).
Studies in which the gene encoding Bim either has been knocked out in murine thymocytes or knocked down in lymphoid cell lines have established the important role of Bim in mediating glucocorticoid-induced apoptosis (21,22). Moreover, the mechanism of Bim induction involves glucocorticoid-mediated repression of a microRNA cluster known to suppress Bim levels in lymphoma cells (23).

Although apoptosis induction by glucocorticoids has been the singular focus of investigators interested in understanding glucocorticoid-induced cell death for the past twenty-five years, we and others have recently documented that dexamethasone also induces macroautophagy (hereafter referred to as autophagy) in lymphocyte cell lines and in primary acute lymphoblastic leukemia cells (24,25). Autophagy is a highly conserved response to metabolic stress in which cellular proteins and organelles are degraded for the maintenance of homeostasis (26,27).

In our investigations of dexamethasone-mediated autophagy, we employed the WEHI7.2 murine T-cell line as the principle system for two main reasons: first, WEHI7.2 cells resemble immature thymocytes in that they are CD4/CD8 positive, and very sensitive to dexamethasone-induced cell death; second, we previously used WEHI7.2 cells in gene expression profiling experiments and therefore have a large database of dexamethasone-regulated genes in this cell line (17,18). In previous work, we documented the induction of autophagy in WEHI7.2 cells by dexamethasone using a variety of methods, including conversion of LC3-I to LC3-II, localization of GFP-LC3 in a punctate pattern, increased degradation of long-lived proteins, and detection of autophagosomes by electron microscopy (25).

In studies reported here we sought to gain insight into the mechanism by which dexamethasone induces autophagy in lymphocytes. Since dexamethasone-induced autophagy is glucocorticoid receptor-mediated (25), we analyzed our microarray database of glucocorticoid-regulated genes for clues. This led us to the current focus on a glucocorticoid-induced gene to which we originally referred to as dexamethasone-induced gene 2, or Dig2. We cloned this gene from an expressed sequence tag identified as glucocorticoid-induced in normal murine thymocytes and in the murine T-cell lines WEHI7.2 cells and S49.1. Northern and Western blot analysis confirmed that Dig2 is rapidly up-regulated in lymphocytes following glucocorticoid treatment. The glucocorticoid antagonist RU486 blocked glucocorticoid-mediated Dig2 induction, indicating that it is mediated through the glucocorticoid receptor. Additionally, Actinomycin D and cyclohexamide also blocked Dig2 induction by glucocorticoids, suggesting a need for de novo transcription and translation (18). In retrospect, Dig2 is identical to the gene referred to as RTP801 or REDD1, described a year earlier as a hypoxia-inducible factor-responsive gene and a developmentally regulated transcriptional target of p63 and p53 (28,29). Thus, for clarity, we are employing the designation Dig2/RTP801/REDD1 in this report.

Significantly, Dig2/RTP801/REDD1 recently was found to be a negative regulator of mTOR signaling (30-33). Dig2/RTP801/REDD1 is proposed to inhibit mTOR by displacing TSC2 (tuberin) from the 14-3-3 binding protein, allowing TSC2 to inhibit mTOR (32). mTOR is a central component of a signaling network that regulates cell growth, size and proliferation (reviewed in reference (34)). Inhibition of mTOR triggers autophagy, although the precise mechanism is unknown (35-38).

In this report we investigated the hypothesis that Dig2/RTP801/REDD1 elevation following dexamethasone treatment contributes to induction of autophagy in thymocytes and murine lymphoma lines. Using RNAi to knock down Dig2/RTP801/REDD1 we are able to partially inhibit glucocorticoid-induced autophagy in WEHI7.2 cells, as measured by LC3 conversion and localization of GFP-LC3 in a punctate pattern, and partially restore mTOR activity. We confirmed these in vitro findings in murine thymocytes after in vivo treatment of wild type and Dig2/RTP801/REDD1 knockout mice. Taken together our findings suggest dexamethasone-mediated induction of autophagy occurs, at least in part, through Dig2/RTP801/REDD1.

**Experimental Procedures**

**Cell Culture and Reagents** The WEHI7.2 murine T-cell lymphoma cell line was cultured in...
Dulbecco’s modified Eagle’s medium supplemented with 2mM L-glutamine, 10% bovine calf serum (Hyclone), and 100µM nonessential amino acids. WEHI7.2 cells were cultured at 1.5x10^5 cells/mL in a humidified 7% CO2 incubator. Stable clones of Bcl-2 overexpressing WEHI7.2 cells were generated and characterized previously (39). Dexamethasone was purchased from Sigma-Aldrich and dissolved in 100% ethanol (vehicle). E64d was purchased from BioMol and pepstatin A from Roche. All other chemicals were from Amresco. 

**Immunoblotting**- Whole cell lysates were obtained by suspending 5 million cells in RIPA buffer (10mM Tris-HCl pH7.6, 150mM NaCl, 5mM EDTA, 1% Triton X-100, 0.1%SDS, 200mM dithiothreitol, 0.5% sodium deoxycholate, and Complete Mini protease inhibitor cocktail (Roche)). Following protein concentration determination using Bradford Reagent (Bio-Rad), equal amounts of protein and sample buffer (50mM Tris-HCl pH6.8, 2% SDS, 100mM dithiothreitol, 10% glycerol and 0.05% bromophenol blue; final concentrations) were separated onto a 15% SDS-PAGE gel, transferred to PVDF membrane and blocked in Tris-buffered saline containing Tween-20 (0.1%) and milk or bovine serum albumin (5%). The following primary antibodies were used: Anti-LC3 (cat# NB100-2220) from Novus Biologicals, anti-phospho p70 S6 kinase (Thr389) and anti- total p70 S6 kinase from Cell Signaling, anti-REDD1 from Protein tech Group Inc, anti-actin antibody from Sigma-Aldrich, and anti-TSC2 (C-20) from Santa Cruz Biotechnology. Quantification of immunoblot signal intensity was performed using ImageJ software and all values were normalized to Actin expression levels.

**RNA interference**- The siGENOME SMARTpools for Dig2/RTP801/REDD1, TSC2 and the negative control siCONTROL non-targeting siRNA pool were purchased from Dharmacon. After suspension in 1X siRNA buffer, SMARTpools were added to cells and electroporation was performed as previously described (40). Cells were incubated for 16 hr after electroporation before the addition of vehicle or dexamethasone.

**Introduction of lentiviral shRNA**- A mouse pLKO.1 lentiviral vector set encoding 5 different shRNAs against Dig2/RTP801/REDD1 (RMM4532-NM_029083) were purchased from Open Biosystems. Dig2/RTP801/REDD1 shRNA vectors were transduced into 293-T cells and virus was harvested 24 hr following transduction. The vectors were then transduced into WEHI7.2 cells overexpressing Bcl-2 and placed under puromycin selection (2µg/mL) as previously described (23). Trypan Blue staining for cell death was performed as previously described (25).

**GFP-LC3 expression and imaging**- WEHI7.2 cells overexpressing Bcl-2 and stably expressing lentiviral vectors of either control or Dig 2/RTP801/REDD1 (KD) shRNA were transiently transfected with the pEGFP-C1-LC3 plasmid, kindly provided by Tamotsu Yoshimori and Noboru Mizushima (41), by electroporation using previously described electroporation conditions (42). After incubation for 16hr, 1µM dexamethasone or vehicle was added to the cells for 4hr at 37ºC. As described in Results, experiments were performed both in the presence and absence of lysosomal protease inhibitors, E64d (5µg/mL) and pepstatin A (10µg/mL). Representative confocal images were acquired with an UltraVIEW VoX spinning disc confocal system (PerkinElmer), which is mounted on a Leica DMI6000B microscope (Leica Microsystems) equipped with a HCX PL APO 100x/1.4 oil immersion objective. GFP-LC3 and DAPI fluorescence were excited using solid state diode lasers (488 nm and 405 nm, respectively) and collected with appropriate emission filters. All confocal images were analyzed using the Velocity software. To quantitatively examine the amount of autophagy, cells were counted based on the fluorescence of GFP-LC3 subcellular distribution, which was scored as either diffuse cytosolic staining or punctate (cells containing 3 or more GFP-LC3 dots). For each experiment over 100 cells were counted per sample. The experiments were performed both with and without inhibitors. Cells were counted by eye using a Zeiss Axiosvert S100 inverted epifluorescent microscope.

**Dig 2/RTP801/REDD1 knockout mice**- Dig 2/RTP801/REDD1 knockout mice were backcrossed onto C57BL6 background (Jackson Labs). Mice were given a 100µL intraperitoneal injection of vehicle (phosphate buffered saline) or dexamethasone sodium phosphate (APP Pharmaceuticals) diluted in vehicle. Four hr after treatment, the thymus gland was isolated as previously described (18). All mice were between...
5 and 7 weeks old. Annexin-V and Propidium Iodide staining was performed as previously described (23). Experiments using mice were approved by the Case Western Reserve University Institutional Animal Care and Use Committee.

Statistics- Prism software was used to perform ANOVA tests on group populations to determine the presence of significance. Two tailed t-tests were used to determine significance between pairs of data. Error bars represent mean ± SE of at least 3 independent experiments.

RESULTS

Since dexamethasone rapidly induces apoptotic cell death in wild type WEHI7.2 cells, we performed experiments reported here in WEHI7.2 derivatives in which Bcl-2 has been overexpressed, thus preventing the complicating factor of cell death. This strategy is based on our earlier observation that Bcl-2 overexpression inhibits dexamethasone-induced apoptosis, but does not inhibit dexamethasone-induced autophagy (25), consistent with evidence that Bcl-2 does not invariably inhibit autophagy (43). We detected dexamethasone-induced elevation of Dig2/RTP801/REDD1 in Bcl-2-positive WEHI7.2 cells (Fig. 1A) by immunoblotting, consistent with the rapid elevation of Dig2/RTP801/REDD1 mRNA following dexamethasone treatment in our earlier studies (18). Although the predicted molecular mass of Dig2/RTP801/REDD1 is 25kD, we and others observe a mobility of 34kD on gel electrophoresis (28,29). Autophagy induction was detected within the same time frame as Dig2/RTP801/REDD1 elevation, based on an increase in LC3-II levels detected by immunoblotting (Fig. 1A). The early time course of autophagy induction in these experiments is consistent with our previous results using time-lapse imaging to demonstrate conversion of GFP-LC3 from a diffuse to punctate pattern within 4 hr of dexamethasone addition to Bcl-2-positive WEHI7.2 cells (25). Furthermore, partial knockdown of Dig2/RTP801/REDD1 either by transient transfection of siRNA (Fig. 1B) or by lentiviral-mediated shRNA (Fig. 1C) inhibited the elevation of LC3-II following dexamethasone treatment. Note that LC3-II elevation following dexamethasone treatment is detected optimally within 4-16 hr after adding dexamethasone to cells (Fig. 1C, lanes 2-4), which is consistent with the timing of Dig2/RTP801/REDD1 induction shown in Fig. 1A.

To verify the findings in Fig. 1, we transiently transfected the same control and Dig2/RTP801/REDD1 knockdown cells used in Figure 1C with a GFP-LC3 expression vector. The conversion of GFP-LC3 from a diffuse to a punctate pattern indicates autophagosome formation (44). Representative confocal images depicting diffuse and punctate GFP-LC3 staining are shown in control and knockdown cells following 4 hr of either vehicle or dexamethasone treatment and co-stained with DAPI (Fig. 2A). To quantify the amount of autophagy, we scored cells based on their appearance as either diffuse or punctate. We monitored the GFP-LC3 pattern 4 hr after vehicle or dexamethasone addition, both in the presence and absence of lysosomal protease inhibitors, pepstatin A (10µg/mL) and E64d (5µg/mL). These inhibitors block the degradation of LC3-II on the inner membrane of autophagosomes and thus differentiate between autophagic flux (the accumulation and degradation of autophagic vesicles) and steady state (which determines only the accumulation of the autophagosomes). This is necessary to distinguish between autophagic activity and an aberration in lysosomal degradation (reviewed in (44)). Dexamethasone treatment increased the percentage of cells with a punctate GFP-LC3 pattern in control shRNA cells in the presence and absence of inhibitors (Figs. 2B & 2C). Knocking down Dig2/RTP801/REDD1 significantly reduced the percentage of cells displaying a punctate GFP-LC3 pattern following dexamethasone treatment, both in the presence and absence of lysosomal protease inhibitors (Figs. 2B & 2C). These findings further substantiate a role for Dig2/RTP801/REDD1 in mediating dexamethasone-induced autophagy.

Previously, we determined that pharmacological inhibition of autophagy increased glucocorticoid-induced apoptosis (25). Since Dig2/RTP801/REDD1 appears to induce autophagy in the Bcl-2 overexpressing WEHI7.2 cells, we next sought to determine whether Dig2/RTP801/REDD1 knockdown altered dexamethasone-induced cell death. Using the same cells as in Figs 1C & 2, we treated control and Dig2/RTP801/REDD1 knockdown cells with
vehicle or dexamethasone for 72 hr. As expected, knockdown of Dig2/RTP801/REDD1 led to increased cell death (Fig. 3). This finding suggests that autophagy prevents or delays cell death following glucocorticoid treatment and corroborates our previous findings that Dig2/RTP801/REDD1 overexpression partially inhibits glucocorticoid-induced apoptosis (18).

Since recent reports suggest Dig2/RTP801/REDD1 inhibits mTOR signaling in other contexts (30-33), we next sought to investigate whether dexamethasone-mediated induction of Dig2/RTP801/REDD1 inhibits mTOR in lymphocytes. As shown in Fig. 4A, dexamethasone treatment of Bcl-2-positive WEHI7.2 cells at concentrations of 10nM or higher for 4 hr inhibited mTOR activity, monitored according to the phosphorylation of S6 kinase (S6K), a typical marker of mTOR activity (34). The same concentration range of dexamethasone induced autophagy, based on LC3-II elevation. As a positive control, we treated cells with rapamycin, which also inhibited mTOR and induced LC3-II elevation. This observation is consistent with earlier evidence that rapamycin induces autophagy by inhibiting mTOR signaling (38).

To determine whether mTOR inhibition in dexamethasone-treated cells is at least partially mediated by Dig2/RTP801/REDD1 elevation, we investigated the effect of Dig2/RTP801/REDD1 knockdown on mTOR activity. We performed the knockdown using both lentiviral-mediated shRNAs and by transient expression of siRNAs, as employed in preceding experiments. In both situations, the elevation of Dig2/RTP801/REDD1 4 hr after dexamethasone addition was reduced substantially, partially preventing dexamethasone-mediated mTOR inhibition, again measured according to the phosphorylation of S6 kinase (Figs. 4B-D). Although we previously demonstrated that glucocorticoids induce autophagy in lymphocytes independent of their Bcl-2 expression level (25), Bcl-2 has been shown to regulate autophagy (45). Dexamethasone treatment of wild type WEHI7.2 cells, which express very low Bcl-2 levels (46), also leads to reduced phosphorylation of S6K (Fig. 4E).

A recent report suggests that upregulation of Dig2/RTP801/REDD1 in hypoxic conditions represses mTOR signaling by activating the TSC1/2 complex, an inhibitor of mTOR (32). To determine whether TSC2 is also important in dexamethasone-mediated inhibition of mTOR, we used siRNA to knockdown TSC2. Knockdown of TSC2, in the absence of further treatment, increased the phosphorylation of S6K, consistent with our previous findings that TSC2 knockdown MEFs (47). In the presence of dexamethasone, knockdown of TSC2 partially repressed dexamethasone-mediated inhibition of phosphorylated S6K (Fig. 4F). These findings suggest that Dig2/RTP801/REDD1 inhibits mTOR in lymphocytes following glucocorticoid treatment. Notably, in the present experiments (Figs 4B & 4C), it was necessary to study cells within a short time-frame following dexamethasone addition when the shRNA activity was sufficient to inhibit substantially Dig2/RTP801/REDD1 induction by dexamethasone, rather than at later time points (i.e., 24 hr in Fig. 4C) where the strong induction of Dig2/RTP801/REDD1 by dexamethasone overpowers the shRNA. Overall, these findings are consistent with a role for Dig2/RTP801/REDD1 elevation in mediating dexamethasone-induced autophagy, at least in part through inhibition of mTOR.

Finally, to determine whether our findings were applicable more broadly, we treated wild type and Dig2/RTP801/REDD1 knockout mice with intraperitoneal injections of dexamethasone sodium phosphate or vehicle. After the treatments, primary thymocytes were isolated and evaluated by immunoblotting. Dig2/RTP801/REDD1 was elevated in dexamethasone-treated wild type mice and absent in knockout mice (Fig. 5A). When treated with a low dose of dexamethasone (25 µg), the absence of Dig2/RTP801/REDD1 partially inhibited LC3-II accumulation, suggesting a reduction in autophagy (Figs. 5A & 5B). To ensure equal delivery of dexamethasone to wild type and Dig2/RTP801/REDD1 knockout thymocytes, we immunoblotted for Txnip (thioredoxin interacting protein), a glucocorticoid primary response gene (48), and observed equivalent induction (data not shown). Consistent with our findings in WEHI7.2 cells, dexamethasone-mediated inhibition of mTOR is abrogated in Dig2/RTP801/REDD1 knockout mice (Fig. 5C). As our evidence suggests Dig2/RTP801/REDD1 prevents or delays cell death in lymphocyte models (Fig. 3)(18), next
we sought to determine if Dig2/RTP801/REDD1 functions similarly in thymocytes. Similar to our previous findings, Dig2/RTP801/REDD1 knockout thymocytes displayed increased cell death following treatment with dexamethasone (Fig. 5D). These findings indicate that Dig2/RTP801/REDD1 contributes to dexamethasone-mediated autophagy and preserves cell survival in primary thymocytes.

**DISCUSSION**

Here we report that dexamethasone-induced autophagy is mediated, in part, through the upregulation of Dig2/RTP801/REDD1. This cytoplasmic protein is highly conserved and ubiquitously expressed. Dig2/RTP801/REDD1 is present throughout development and is upregulated rapidly in multiple cell types in response to cell stress induced by hypoxia, DNA-damage and starvation (28,29,33,49).

Our laboratory previously determined that Dig2/RTP801/REDD1 mRNA was increased markedly in lymphocytes within 30 min following treatment with the glucocorticoid hormone dexamethasone or the ER stress inducers tunicamycin and thapsigargin (18). The mechanism of induction by dexamethasone is unknown. Although it is glucocorticoid receptor mediated, we did not find evidence that Dig2/RTP801/REDD1 is a direct transcriptional target of the glucocorticoid receptor (18). Thus, the induction of Dig2/RTP801/REDD1 appears to be an indirect effect of dexamethasone, perhaps mediated through ER stress. Based on a limited number of reports, it appears that elevation of Dig2/RTP801/REDD1 can favor either cell survival or cell death, depending on cellular context (29,50). In the case of dexamethasone-treated lymphocytes, Dig2/RTP801/REDD1 elevation appears to favor cell survival, as inhibition of Dig2/RTP801/REDD1 increases cell death in WEHI7.2 cells and primary thymocytes (Figs. 3 and 5D) and previous work from our laboratory demonstrates that its overexpression inhibits cell death (18).

Autophagy is generally a survival response to cell stress and is often mediated by mTOR inhibition. Based on prior reports indicating that Dig2/RTP801/REDD1 is a negative regulator of mTOR (30-33,49,51), we undertook the present study to determine whether Dig2/RTP801/REDD1 mediates dexamethasone-induced autophagy. Using two RNAi methods, we provide evidence that knocking-down Dig2/RTP801/REDD1 partially blocks the induction of autophagy by dexamethasone in WEHI7.2 cells (Figs. 1, 2 & 4). Importantly, we assessed autophagy progression in both the presence of and absence of lysosomal inhibitors (Fig. 2). In the process of characterizing Dig2/RTP801/REDD1 function, we also determined that it contributes to the inhibition of mTOR, as measured by phosphorylation of S6K, a downstream target of mTOR (Fig. 4)(36). This is consistent with a previous study demonstrating that in muscle cells, Dig2/RTP801/REDD1 elevation by dexamethasone represses mTOR signaling (51). DeYoung et al. propose Dig2/RTP801/REDD1 functions by inhibiting the interaction of TSC2 with 14-3-3, thereby promoting assembly of the TSC1/TSC2 complex, which inhibits mTOR (32). Our findings are consistent with this mechanism as dexamethasone-mediated Dig2/RTP801/REDD1 elevation induces autophagy, at least in part, through mTOR. Also, knockdown of TSC2 suggests Dig2/RTP801/REDD1 function requires TSC2 (Fig. 4).

Additionally, we assessed whether Dig2/RTP801/REDD1 contributes to dexamethasone-mediated autophagy in primary murine thymocytes using the previously characterized Dig2/RTP801/REDD1 knockout mouse (50). When we treated thymocytes with dexamethasone, absence of Dig2/RTP801/REDD1 partially inhibited autophagy induction (Fig. 5), similar to the RNAi-mediated knockdown of Dig2/RTP801/REDD1 in WEHI7.2 cells. It is important to note that we used relatively low doses of dexamethasone when assessing autophagy induction. When we treated mice with high doses of dexamethasone, however, the induction of autophagy was less dependent on Dig2/RTP801/REDD1 (data not shown). This is likely because primary thymocytes, which lack Bcl-2, are very sensitive to glucocorticoid-induced apoptosis and dexamethasone can induce autophagy through multiple mechanisms.

In our investigations of autophagy, we have noticed that different LC3 antibodies, as well as different lots of the same LC3 antibody,
variably detect LC3-I and LC3-II. In some instances, we observe similar affinity of LC3 antibodies to LC3-I and LC3-II and in others, LC3-I is difficult to observe on immunoblot. This phenomenon is also observed by other groups and mentioned in several review articles on the measurement of autophagy (44,52). These articles come to the conclusion that the best way to quantify autophagy by LC3 immunoblotting is to, 1) always compare treated samples to an untreated control and 2) use the ratio of LC3-II/Actin for quantification.

Considering the broad and diverse actions of glucocorticoids, it is of no surprise that multiple mechanisms induce autophagy. Indeed, our laboratory also has described how glucocorticoid-mediated inhibition of the Src family kinase Fyn and consequently a reduction in inositol 1,4,5 triphosphate (IP3)-mediated calcium signals, contributes to autophagy through the inhibition of mTOR (53). Others have reported that glucocorticoids induce autophagy through the inhibition of AKT activity (24). These previous studies focused on autophagy induction by glucocorticoids at later time points (i.e. 16 to 36 hr) than the present study. Fyn knockdown does not alter glucocorticoid-mediated Dig2/RTP801/REDD1 induction and we do not observe a decrease in AKT phosphorylation following glucocorticoid treatment in our cell system (data not shown). Therefore, we believe that the current findings represent an independent and rapid mechanism through which glucocorticoids induce autophagy.

Overall, our data provide novel insight into the mechanism of glucocorticoid-mediated autophagy and this report is, to the best of our knowledge, the first to directly demonstrate that Dig2/RTP801/REDD1 elevation induces autophagy. These findings build on previous reports indicating that Dig2/RTP801/REDD1 is a pro-survival stress response (18,29,50). Thus, therapeutically targeting autophagy may increase the therapeutic potential of glucocorticoids in hematologic malignancies.

Acknowledgements
We thank Tamotsu Yoshimori and Noboru Mizushima for providing us with LC3 cDNA and Mark Jackson for suggestions regarding lentivirus shRNA. This work was supported by NIH grants RO1 CA42755 and CA85804 (CWD), RO1 AG031903 (SM), T32HL007147 (SJS and JKM) and T32 GM007250 (JKM). This research was also supported in part by the Ohio Center for Innovative Immunosuppressive Therapeutics grant, which maintains the spinning disk confocal microscope in the Department of Dermatology’s Morphology Core Facility. The authors have nothing to disclose.

References

**FIGURE LEGENDS**

Fig. 1: Dig2/RTP801/REDD1 knockdown by shRNA and siRNA partially inhibits dexamethasone-induced autophagy. A. WEHI7.2 cells overexpressing Bcl-2 were treated with vehicle (0.1% ethanol) for 8 hr or 1µM dexamethasone for 2, 4, and 8 hrs. Protein lysates were probed for Dig2/RTP801/REDD1, LC3 and Actin (loading control). B. Bcl-2-positive WEHI7.2 cells were transiently transfected with 1000nM non-targeting siRNA (NT) or Dig2/RTP801/REDD1 siRNA (KD) and after 16 hr were treated with 1µM dexamethasone or vehicle for 4 hr prior to immunoblotting for Dig2/RTP801/REDD1 and LC3. C. Bcl-2-positive WEHI7.2 cells were stably transduced with lentivirus encoding either control shRNA or shRNA directed at Dig2/RTP801/REDD1 (Dig 2 shRNA). Cells were then treated with 1µM dexamethasone or vehicle for the times indicated and levels of Dig2/RTP801/REDD1 and LC3 were
assessed by immunoblotting. Values represent normalized levels of LC3-II expression relative to Actin. All immunoblots are representative of multiple independent experiments.

Fig. 2: GFP-LC3 analysis confirms that Dig2/RTP801/REDD1 knockdown inhibits dexamethasone-induced autophagy. Stably transduced lentiviral vector control (control shRNA) and Dig2/RTP801/REDD1 knockdown (Dig 2 shRNA) WEHI7.2 cells (as in Figure 1C) were transiently transfected with GFP-LC3 for microscopic assessment of autophagosome formation. A. Representative confocal images for GFP-LC3 alone and GFP-LC3 with DAPI; Scale bar, 10µm. B & C. The percentage of punctate cells (3 or more punctate dots) was scored blind to the sample identity after 4 hr treatment of vehicle (0.1% ethanol) or 1µM dexamethasone in the presence (B) or absence (C) of lysosomal inhibitors (pepsatin A 10µg/mL and E64d 5µg/mL). Error bars represent mean ± SE of at least 3 independent experiments. * indicates p<0.05, ** indicates p<0.01 and n.s. indicates p>0.05.

Fig. 3: Dig2/RTP801/REDD1 knockdown increases dexamethasone-mediated cell death. Stably transduced lentiviral vector control (control shRNA) and Dig2/RTP801/REDD1 knockdown (Dig 2 shRNA) Bcl-2 overexpressing WEHI7.2 cells (as in Fig. 1C) were treated with vehicle (0.1% ethanol) or 1µM dexamethasone for 72 hr. Cell death was assayed by trypan blue staining. Error bars represent mean ± SE of at least 3 independent experiments. * indicates p<0.01 between control and Dig2/RTP801/REDD1 knockdown dexamethasone-treated samples.

Fig. 4: Inhibition of mTOR signaling by Dig2/RTP801/REDD1 in dexamethasone-treated WEHI7.2 cells. A. Protein lysates of WEHI7.2 cells overexpressing Bcl-2 were collected following no treatment (untreated) or 4 hours of treatment with vehicle (0.1% ethanol), rapamycin (20nM) or a dose response of dexamethasone in the presence of lysosomal inhibitors where indicated (pepsatin A 10µg/mL and E64d 5µg/mL). Immunoblots were probed for Dig2/RTP801/REDD1, LC3, Thr389 phosphorylated S6K (pS6K) and total S6K. Values represent normalized levels of LC3-II expression relative to Actin. B. Bcl-2-positive WEHI7.2 cells were transiently transfected with non-targeting control siRNA (NT) and Dig2/RTP801/REDD1 siRNA (KD), followed by treatment with vehicle (0.1% ethanol) or 1µM dexamethasone for 4 hr. mTOR activity was assessed by immunoblotting for Thr389 phosphorylated S6K (pS6K) and total S6K. C. Bcl-2 positive WEHI7.2 cells stably transduced with lentiviral shRNA control and Dig2/RTP801/REDD1 shRNA were treated with vehicle (0.1% ethanol) or 1µM dexamethasone for both 4 and 24 hr. Immunoblotting was performed as in panel B. D. Densitometry of immunoblot signal intensity from 4 hr treatments was used to quantify relative expression levels of pS6K. Signal intensity was normalized to the signal intensity of Actin. E. Bcl-2-positive WEHI7.2 cells were transiently transfected with 1000nM non-targeting siRNA (NT) or TSC2 siRNA (KD) and after 16 hr were treated with 1µM dexamethasone or vehicle for 4 hr prior to immunoblotting for Dig2/RTP801/REDD1, pS6K and total S6K. F. Wild type WEHI7.2 cells were treated with 1µM dexamethasone or vehicle for 4 hr prior to immunoblotting for Dig2/RTP801/REDD1, pS6K and total S6K. Error bars represent mean ± SE of at least 3 independent experiments. All immunoblots are representative of multiple independent experiments. * indicates p<0.05 between control and Dig2/RTP801/REDD1 knockdown dexamethasone-treated samples.

Fig. 5: Dig2/RTP801/REDD1 knockout mouse thymocytes display partial inhibition of dexamethasone-induced autophagy. A-C. Primary thymocytes were isolated from wild type and Dig2/RTP801/REDD1 knockout mice following intraperitoneal injections of vehicle (phosphate buffered saline) or dexamethasone sodium phosphate for 4 hr. Mice were treated with vehicle or 25µgs dexamethasone sodium phosphate. A. Immunoblots for Dig2/RTP801/REDD1 and LC3. B. The immunoblot signal for LC3-II was quantified using densitometry relative to actin and normalized to vehicle-treated mice for 2 independent experiments. C. Thr389 phosphorylated S6K (pS6K) and total S6K. D. Wild type and Dig2/RTP801/REDD1 knockout mice were given intraperitoneal injections of vehicle or 200µgs dexamethasone for 24 hr. Primary thymocytes were isolated and cell death was assayed using Annexin-V/propidium iodide staining and was quantified using flow cytometry. Error bars represent mean ± SD. All immunoblots are representative of multiple independent experiments.
Figure 1

**A**

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Dig 2/ RTP801/ REDD1

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Actin

**B**

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>VEH</th>
<th>1μM Dex</th>
</tr>
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<tbody>
<tr>
<td>NT</td>
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<td></td>
</tr>
<tr>
<td>KD</td>
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<table>
<thead>
<tr>
<th>Dig 2/ RTP801/ REDD1</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC3 I</td>
</tr>
<tr>
<td>LC3 II</td>
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</table>

<table>
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<tr>
<th>1.00</th>
<th>1.00</th>
<th>2.16</th>
<th>0.51</th>
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Actin

**C**

<table>
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<tr>
<th>Time (hr)</th>
<th>Control shRNA</th>
<th>Dig 2 shRNA</th>
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<table>
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<tbody>
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<tr>
<td>LC3 II</td>
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<table>
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<th>5.42</th>
<th>5.30</th>
<th>1.49</th>
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</thead>
</table>

Actin

Figure 1
Figure 2

A

GFP-LC3  GFP-LC3 & DAPI

VEH

Dex

Dig 2 shRNA

VEH

Dex

B

Control shRNA  Dig 2 shRNA

+ Lysosomal Inhibitors

VEH  Dex

punctate cells (%)

C

Control shRNA  Dig 2 shRNA

- Lysosomal Inhibitors

VEH  Dex

punctate cells (%)

*  **

n.s.
Figure 3

VEH
Dex

Control shRNA
Dig 2 shRNA

Cell Death (%)
Figure 5

A

<table>
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<tr>
<th></th>
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<th>Dig 2 KO</th>
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<tbody>
<tr>
<td>25μg Dex</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Dig 2/</td>
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<td></td>
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<tr>
<td>RTP801/REDD1</td>
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<tr>
<td>LC3 I</td>
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<tr>
<td>Actin</td>
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B

LC3-II Expression Fold Change

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<tr>
<td>25μg Dex</td>
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C

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<tr>
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<tr>
<td>pS6K</td>
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</tbody>
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D

Log PI vs. Log Annexin V

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</thead>
<tbody>
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<td></td>
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<tr>
<td>Dex</td>
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Figure 5
VOLUME 285 (2010) PAGES 34960–34971  
DOI 10.1074/jbc.A110.150839

**Starch binding domain-containing protein 1/genethonin 1 is a novel participant in glycogen metabolism.**

Sixin Jiang, Brigitte Heller, Vincent S. Tagliabracci, Lanmin Zhai, Jose M. Irimia, Anna A. DePaoli-Roach, Clark D. Wells, Alexander V. Skurat, and Peter J. Roach

Since publication of this article, we have become aware of an error in the experimental protocol to visualize LAMP1 in cells by immunofluorescence. Specifically, the wrong antibodies were used, so assessments of LAMP1 distribution are not reliable. We did additional experiments, including analysis of the distribution of LAMP2, which is found in the same compartments as LAMP1. We observed no co-localization of LAMP2 and Stbd1. However, the primary conclusion of the study, the link between Stbd1 and glycogen metabolism, is unaffected, as is our hypothesis that Stbd1 anchors glycogen to membranes and may be involved in its localization and trafficking within the cell.

VOLUME 286 (2011) PAGES 34959–34975  
DOI 10.1074/jbc.A110.187666

**Liver-specific inducible nitric-oxide synthase expression is sufficient to cause hepatic insulin resistance and mild hyperglycemia in mice.**

Shohei Shinozaki, Cheol Soo Choi, Nobuyuki Shimizu, Marina Yamada, Minhye Kim, Ting Zhang, Goshi Shiota, H. Henry Dong, Young-Bum Kim, and Masao Kaneki

Dr. Goshi Shiota was inadvertently omitted from the author list. The correct author list is shown above. Dr. Shiota’s affiliation is as follows: Division of Molecular and Genetic Medicine, Department of Genetic Medicine and Regenerative Therapeutics, Graduate School of Medicine, Tottori University, Yonaga 683-8504, Japan.

VOLUME 286 (2011) PAGES 30181–30189  
DOI 10.1074/jbc.A111.245423

**Glucocorticoid elevation of dexamethasone-induced gene 2 (Dig2/RTP801/REDD1) protein mediates autophagy in lymphocytes.**

Jason K. Molitoris, Karen S. McColl, Sarah Swerdlow, Mieko Matsuyama, Minh Lam, Terri H. Finkel, Shigemi Matsuyama, and Clark W. Distelhorst

The grant information footnote should read as follows. This work was supported, in whole or in part, by National Institutes of Health Grants R01 CA42755 and CA85804 (to C. W. D.), R01 AG031903 (to S. M.), T32 HL007147 (to S. S. and J. K. M.), and T32 GM007250 (to J. K. M). The “Acknowledgments” should read as follows. The Dig2/RTP801 knock-out mice were obtained from Quark Pharmaceuticals, Inc., for whom they were exclusively generated at Lexicon. We thank Tamotsu Yoshimori and Noboru Mizushima for providing LC3 cDNA and Mark Jackson for suggestions regarding lentiviral shRNA. This work was supported in part by an Ohio Center for Innovative Immunosuppressive Therapeutics grant, which maintains the spinning disk confocal microscope in the Morphology Core Facility of the Department of Dermatology, Case Western Reserve University.

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Glucocorticoid elevation of dexamethasone-induced gene 2 (Dig2/RTP801/REDD1) mediates autophagy in lymphocytes
Jason K. Molitoris, Karen S. McColl, Sarah Swerdlow, Mieko Matsuyama, Minh Lam, Terri H. Finkel, Shigemi Mastsuyama and Clark W. Distelhorst

J. Biol. Chem. published online July 6, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M111.245423

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