Skp1-Cul1-F-box Ubiquitin Ligase (SCFβTrCP)-mediated Destruction of the Ubiquitin-specific Protease USP37 during G2-phase Promotes Mitotic Entry*

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Background: USP37 regulates S-phase progression and is degraded in late M/G1.
Results: USP37 undergoes biphasic degradation during G2 and late M/G1.
Conclusion: SCFβTrCP and APC-Cdh1 coordinately regulate USP37 during the cell cycle.
Significance: Precise regulation of USP37 activity is required for cell cycle progression.

Ubiquitin-mediated proteolysis is a key regulatory process in cell cycle progression. The Skp1-Cul1-F-box (SCF) and anaphase-promoting complex (APC) ubiquitin ligases target numerous components of the cell cycle machinery for destruction. Throughout the cell cycle, these ligases cooperate to maintain precise levels of key regulatory proteins, and indirectly, each other. Recently, we have identified the deubiquitinase USP37 as a regulator of the cell cycle. USP37 expression is cell cycle-regulated, being expressed in late G1 and ubiquitinated by APC-Cdh1 in early G1. Here we report that in addition to destruction at G1, a major fraction of USP37 is degraded at the G2/M transition, prior to APC substrates and similar to SCFβTrCP substrates. Consistent with this hypothesis, USP37 interacts with components of the SCF in a βTrCP-dependent manner. Interaction with βTrCP and subsequent degradation is phosphorylation-dependent and is mediated by the Polo-like kinase (Plk1). USP37 is stabilized in G2 by depletion of βTrCP as well as chemical or genetic manipulation of Plk1. Similarly, mutation of the phospho-sites abolishes βTrCP binding and renders USP37 resistant to Plk1 activity. Expression of this mutant hinders the G2/M transition. Our data demonstrate that tight regulation of USP37 levels is required for proper cell cycle progression.

The APC recognizes substrates (e.g. cyclins, securin, Geminin) containing one or more destruction-targeting motifs (degrons), primarily the destruction box (D-box) RXRX(L)2, and the KEN box (3). The ability of the APC to recognize these degrons is conferred, at least in part, by the adaptor/activator proteins Cdc20 and Cdh1 (4). Rigid control of the APC is achieved by a variety of mechanisms. The expression of Cdc20 and Cdh1 as well as their interactions with the APC are cell cycle-dependent with APC-Cdh1 active primarily in late mitosis through G1, and APC-Cdc20 active during mitosis (5, 6). The APC exhibits autonomous regulation by targeting its activators and its cognate E2s for destruction (7, 8). In addition, there are also a number of direct inhibitors of the APC. The bulk of APC-Cdh1 activity is kept in check from G1-M by the inhibitor Emi1.

Similar to the APC, SCF ligases are denoted by the substrate-adapting F-box protein. SCFSkp2 and SCFβTrCP have prominent roles in the cell cycle (9). In contrast to the APC, SCF complex activity toward substrates is largely mediated by the cell cycle- or stimulus-dependent phosphorylation of substrates. SCFβTrCP, for example, recognizes phosphorylated serines in the DSGXXS motif in its substrates (e.g. Emi1, Wee1, Claspin, Cdc25A) (9–14). Intriguingly, many of these substrates are phosphorylated by the APC substrate Plk1 (12, 14–16). There is also significant cross-talk between the APC and SCF ligases. For example, the ligases act in tandem to regulate the levels of a number of critical cell cycle regulators, including Cdc25A and Claspin (9, 10, 12, 17–19). In addition, APC-Cdh1 controls SCF activity by targeting Skp2, whereas SCFβTrCP regulates APC activity by targeting Emi1 for destruction (11, 13, 20, 21).

Given the critical function of ubiquitin in control of the cell cycle, deubiquitinating enzymes are expected to play central roles as well. Indeed, several deubiquitinating enzymes have been implicated in the cell cycle. Recently, we have identified the deubiquitinating enzyme USP37 as a regulator of the G1/S transition (22). USP37 regulates S-phase entry at least in part by enhancing cyclin A stability and accumulation (22). Consistent with its regulation of this key cell cycle transition, USP37 is required for zebrafish development (23). USP37 is regulated by the oncogenic transcription factor E2F1, and its expression is increased in several cancers (24–28). Increased USP37 expression is associated with poor prognosis in non-small cell lung
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cancer (29). The phenotypes associated with aberrant USP37 activity are unlikely to be explained by its effect on cyclin A and have prompted us to explore USP37 biology further.

Here we report that destruction of USP37 is biphasic. USP37 is destroyed in G2 by the concerted actions of Plk1 and SCFβTrCP, whereas APC^Cdh1 targets the remaining pool at mitotic exit. By expressing UPS37 mutants that are resistant to SCFβTrCP-mediated ubiquitination, we demonstrate that destruction of this pool is required for mitotic entry. Importantly, this destruction event highlights the existence of additional substrates whose destruction is required for the G2/M transition.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**

HeLa, 293T, U2OS, and T98G cells were obtained from ATCC and maintained in DMEM supplemented with 10% FBS. HeLa and 293T cells were synchronized as described (30). RO-3306 or nocodazole were added 5 h after release from thymidine. T98G cells were synchronized by incubation in DMEM without FBS for 72 h and stimulated to re-enter the cell cycle by the addition of 20% FBS. Cells were transfected with TransIT-LT1 (Mirus Bio) or RNAiMAX (Invitrogen) per the manufacturer’s instructions. Where indicated, cells were treated with 100 ng/ml nocodazole, 10 μM RO-3306 (EMD Millipore), 200 nM BI2536, and 10 μM MG132 (Boston Biochem).

**Antibodies**

The USP37 and Em11 antibodies were described previously (22, 31). Anti-MYC (9E10) was produced at Lerner Research Institute (LRI). Commercial antibodies were as follows: Santa Cruz Biotechnology, cyclin B (GNS1), cyclin A (H-432), Skp2 (H-435); Sigma, actin (AC-15), Claspin; Thermo Scientific, Cdh1 (DH01); Boston Biochem, UbcH10; Covance, HA.11; BD Biosciences, Aurora B (AIM1), Nek2; Enzo Life Sciences, Plk1 (3D8); Cell Signaling, βTrCP (D13F10), pHistone H3, pRb, Cdk1 pY15; Invitrogen, p27, and Securin (pituitary tumor transforming gene 1); and MBL International, Cdc20.

**Plasmids and Recombinant Proteins**

USP37 was subcloned into pDEST-CS2-MYC_6 and pDEST-GEX-6P1 using Gateway technology (Invitrogen). Mutants were generated by the QuikChange mutagenesis strategy. Additional plasmids were described previously (13, 16). His_6-Ubiquitin was generated by PCR and cloned into pCDNA5/FRT/TO (Invitrogen). Recombinant and in vitro translated protein were produced as described (30) except that USP37 was produced in wheat germ rather than rabbit reticulocyte lysate.

**Western Blotting and Immunoprecipitation**

Cell extracts were generated in EBC buffer (50 mM Tris (pH 8.0), 120 mM NaCl, 1% Nonidet P-40, 1 mM DTT, 25 mM β-glycerophosphate, 5 mM NaF, 1 mM NaVO_4, and leupeptin, pepstatin, and chymotrypsin, each at 10 μg/ml). For immunoprecipitation, equal amounts of cell lysates were incubated with the indicated antibodies for 2–12 h and washed in EBC buffer including inhibitors. Immunoprecipitation samples or equal amounts of whole cell lysates were resolved by SDS-PAGE, transferred to PVDF membranes (Millipore) probed with the indicated antibodies, and visualized with the LI-COR Odyssey infrared imaging system.

**Ubiquitination**

*In Vivo*—293T cells were transfected with a 1:1:2 ratio of His_6-Ub-MYC-USP37:HA-βTrCP between thymidine blocks. 10 μM MG132 was added during the last 10–12 h of culture. Lysates were generated as above, except that 2 mM N-ethylmaleimide was added to inactivate deubiquitinating enzymes and DTT was omitted. Equal amounts of lysates were adjusted to 1.5% SDS and boiled for 10 min. Lysates were cooled to room temperature, and ubiquitinated proteins were purified with Ni^{2+}-agarose and processed as above.

*In Vitro*—The in vitro procedure was essentially as described (32) except that FLAG-βTrCP was used, UbcH3 was the sole E2, and USP37 substrates were translated in vitro.

**Flow Cytometry and Immunofluorescence**

Analyses were performed as described (30, 33).

**RESULTS**

**USP37 Is Targeted by APC^Cdh1 in G1**—Previously, we demonstrated that APC^Cdh1 targets USP37 for destruction (22). USP37 is modified by Lys11-linked polyubiquitin chains in late mitosis/G1 and is destroyed with similar kinetics to other APC^Cdh1 substrates (22) (supplemental Fig. S1, A and B). However, the requirement for Cdh1 for destruction of USP37 in G1 was not determined. We therefore depleted Cdh1 or Cdc20 in thymidine-nocodazole synchronized HeLa cells and followed the kinetics of USP37 destruction (supplemental Fig. S1C). As expected, USP37 levels remained stable in cells depleted of Cdh1, but not Cdc20. To confirm that this was due to direct activity of Cdh1 toward USP37 and not a cell cycle defect, we performed knockdown experiments in T98G cells synchronized in G0 by serum starvation. Cells were transfected with siRNAs at serum stimulation to prevent additional Cdh1 expression. Consistent with our HeLa cell data, depletion of Cdh1 in T98G cells resulted in premature accumulation of USP37 and cyclin A (supplemental Fig. S1D).

**USP37 Is Unstable in G2**—During the course of the above experiments, we observed that the levels of USP37 in nocodazole-arrested cells were lower than those in thymidine-arrested cells. This observation was surprising in light of the results above and our previous study (22) as APC^Cdh1 is thought to be inactive from G1/S through anaphase. We therefore examined USP37 levels in HeLa cells synchronized by a double-thymidine block as they progressed from S-phase through early G1 (Fig. 1A). Cell cycle progression was monitored by flow cytometry (supplemental Fig. S2A). USP37 levels steadily declined as cells progressed through mitosis and into G1 (8–12 h) (Fig. 1, A and B, supplemental Fig. S2A). The rate of degradation slowed as cells progressed through mitosis and into G1 (6–8 h) (Fig. 1, A and B, supplemental Fig. S2A). We compared the decline of USP37 levels with substrates of APC^Cdc20 and APC^Cdh1 as well as the SCFβTrCP substrate Emi1. Because APC substrates decline
in two activator-dependent waves, we analyzed USP37 destruction in two phases and set the level of all proteins to 1 at the first time point of each phase (i.e. 6 and 8 h) (Fig. 1, C and D). As cells transited G2 through the early stages of mitosis, USP37 levels declined by 50% similar to Emi1, whereas the APCCdc20 substrates remained stable (Fig. 1C, supplemental Fig. S2A). USP37 degradation slowed as APCCdc20 substrates were degraded and then paralleled the degradation of APCCdh1 substrates (Fig. 1C and D, supplemental Fig. S2A). The apparent stabilization of USP37 during the period of APCCdc20 substrate destruction is in agreement with the existence of a pool of USP37, which remains stable in nocodazole (Fig. 1G, supplemental Fig. S1, A–C). The timing of the second wave of destruction is consistent with the Cdh1-dependent destruction of this mitosis-stable pool of USP37 (supplemental Fig. S1C). We confirmed that this was not an artifact of thymidine synchronization by examining USP37 levels in quiescent T98G cells stimulated to enter the cell cycle by serum addition (Fig. 1E). Flow cytometry analysis indicated that destruction of USP37 begins in G2 (supplemental Fig. S2B). Similar results were obtained in HeLa cells released from a nocodazole arrest (Fig. 1F). USP37 levels began to decline prior to APC substrates in both of these populations as well (Fig. 1, E and F). Together these data indicate that a pool of USP37 is degraded in G2. To test this hypothesis, we examined USP37 levels in T98G cells treated in late S/G2 with the Cdk1 inhibitor (RO-3306) to prevent mitotic entry (34). Indeed,
The presence of βTrCP and induced the accumulation of ubiquitinated forms (Fig. 3C, left panels). We confirmed that these were ubiquitin-USP37 conjugates by purifying ubiquitinated proteins from the denatured lysates and probing for MYC-USP37 (Fig. 3C, right panel). Finally, we tested the requirement for βTrCP in the degradation of USP37 in G2. Serum-starved T98G cells were depleted of βTrCP via siRNA oligonucleotides known to target both βTrCP1 and βTrCP2 (supplemental Fig. S3A), and USP37 levels were monitored after serum stimulation (35, 37). In control siRNA transfected cells, USP37 levels peaked at 20 h and began to drop at 24 h. In contrast, USP37 levels in siβTrCP transfected cells remained stable through the end of the experiment (32 h) (Fig. 3D). As previously reported, βTrCP depletion resulted in the accumulation of cyclins A and B (supplemental Fig. S3B) (11). Together these results indicate that USP37 is a βTrCP substrate.

To confirm that USP37 is directly regulated by SCFβTrCP, we sought to identify the degron mediating its targeting by βTrCP. The consensus βTrCP recognition sequence is DSGXXS, where both serine residues are phosphorylated (9). Several variants of this phospho-degron have been identified (Fig. 4B) (9). Examination of the USP37 sequence revealed no consensus degrons, but instead identified multiple sequences resembling noncanonical degrons (Fig. 4, A and B). We generated a series of N- and C-terminal deletion mutants to identify sequences that direct USP37 degradation and tested their ability to bind βTrCP (Fig. 4A, supplemental Fig. S3C). The deletion panel was designed to maintain individual structural motifs, termed Boxes 1–6, within the catalytic domain of USP family members that have been identified in structural models of USP37 (38). The pattern of βTrCP interaction with these fragments highlighted a unique insertion between Boxes 4 and 5 of the USP37 catalytic domain that contains three ubiquitin-interacting motifs as well as the APCCdhl-targeting KEN box (Fig. 4A) (22). The ubiquitin-interacting motif insertion contains three potential βTrCP binding sites, (Fig. 4B). An additional deletion mutant that bisects the ubiquitin-interacting motif insertion further highlighted residues 756–888, which contain two potential binding motifs. We created S → A mutants of the likely phospho-sites within these two motifs (Fig. 4B). USP37 S858A, but not the S790A mutant, exhibits weakened binding to βTrCP (Fig. 4C). Consistent with direct targeting of USP37 by SCFβTrCP, USP37 S858A is resistant to βTrCP-driven degradation (Fig. 4D).

**Plk1 Triggers the SCFβTrCP-mediated Destruction of USP37**—The Plk1 kinase is a key regulator of the G2/M transition and functions in part by phosphorylating βTrCP recognition sites in proteins that must be destroyed for mitotic entry/progression (e.g. Emi1, Wee1) (14, 16). We therefore hypothesized that Plk1 might regulate the destruction of USP37 as well. Indeed, Ser-858 is highly conserved and lies within a consensus Plk1 phosphorylation motif, (D/N/E/Y)X(S/T) (supplemental Fig. S3D) (39). We found that USP37 and Plk1 were able to interact in cells, confirming the likelihood that Plk1 plays a role in USP37 degradation (Fig. 5A). We then asked whether manipulating Plk1 activity would affect USP37 levels in cells. Expression of Plk1 reduced levels of coexpressed USP37, whereas the dominant-negative Plk1 K82R caused an increase in USP37 lev-
els relative to control cells (Fig. 5B). We then asked whether Plk1 could stimulate βTrCP-mediated destruction of USP37. Indeed, expression of both Plk1 and βTrCP resulted in a strong reduction in USP37 levels that was rescued by proteasome inhibition (Fig. 5C). We then asked whether Plk1 activity is required for the destruction of USP37. We transfected serum-starved T98G cells with siRNA targeting Plk1, which prevented the expression of the kinase upon cell cycle entry. In control populations, USP37 exhibited a steady decline after 24 h, as Plk1 levels increased. USP37 levels remained high in the Plk1-depleted populations through 32 h (Fig. 5D). We further confirmed the involvement of Plk1 by inhibiting its function in thymidine-synchronized U2OS cells with the small molecule BI2536 (supplemental Fig. S3E). Consistent with the siRNA results, inhibition of Plk1 prevented the degradation of USP37 as cells approached mitosis.

The ability of Plk1 to regulate USP37 stability suggests that it should modulate the interaction of USP37 with βTrCP. We tested this model with an in vitro pulldown assay. Recombinant GST-USP37 was utilized to capture in vitro translated βTrCP. As expected, in the absence of phosphorylation, USP37 was unable to interact with βTrCP (Fig. 5E, lane 2). Surprisingly, the addition of recombinant Plk1 had little, if any, effect on the ability of USP37 to bind βTrCP (Fig. 5E, lanes 3 and 4). However, efficient interaction of Plk1 with its substrates requires a priming phosphorylation event, which is frequently mediated by cyclin-dependent kinases (40, 41). Cdk2 in complex with either cyclin A or cyclin E is able to phosphorylate USP37 (22). We reasoned that phosphorylation by these kinases may promote phosphorylation by Plk1 and βTrCP binding. Neither cyclin A-Cdk2 nor cyclin E-Cdk2 induced a strong interaction between USP37 and βTrCP (Fig. 5E, lanes 5–8). However, Plk1

### FIGURE 3. SCFβTrCP regulates USP37 levels.
A, MYC-USP37 was transfected into 293T cells, and levels of the tagged proteins were examined after 48 h. B, MYC-USP37 was transfected into 293T cells synchronized by double thymidine block. C, cells were treated as in B, and His-ubiquitin was included in the transfection mix. Cells were treated with 10 μM MG132 for the last 12 h. Ubiquitinated proteins were purified by Ni²⁺ affinity. Extracts and pulldowns (PD) were probed for the indicated proteins. D, T98G cells were transfected with control or βTrCP-targeting siRNAs during serum starvation and stimulated to enter the cell cycle. The indicated proteins were analyzed throughout the cell cycle.

### FIGURE 4. Identification of the βTrCP degron.
A, schematic representation of USP37. Subdomains of the catalytic domain, KEN-box 3, the ubiquitin-interacting motif (UIM) insertion, and potential βTrCP degrons are indicated. The panel of MYC-USP37 deletion mutants and their ability to bind HA-βTrCP are depicted. B, the consensus sequences of the βTrCP degron, canonical and noncanonical degrons, and potential USP37 degrons are depicted. USP37 degron mutants are indicated. C, the ability of the USP37 degron mutants to interact with HA-βTrCP was tested as in Fig. 2. Vec, vector; IP, immunoprecipitate; D, the ability of βTrCP to induce destruction of USP37 and the S858A mutant was determined as in Fig. 3B.
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In the presence of either cyclin-Cdk2 complex induced a strong interaction between USP37 and βTrCP (Fig. 5E, lanes 9 and 10). Importantly, the S858A mutation dramatically reduced the ability of Plk1 to induce βTrCP binding (Fig. 5F). We then confirmed that Plk1-mediated, Ser-858-dependent binding to βTrCP was required for ubiquitination by the SCF by performing in vitro ubiquitination assays (Fig. 5G). Indeed, ubiquitination of USP37 S858A by SCFβTrCP was drastically reduced in comparison with wild type. Taken together, these results identify Plk1 as a novel USP37-interacting and regulatory protein.

**Destruction of USP37 by SCFβTrCP in G2. Is Required for Mitotic Entry**—We next sought to confirm that SCFβTrCP and Plk1 were mediating destruction of USP37 in G2. We asked whether βTrCP or Plk1 was required for USP37 destruction in T98G cells stimulated to enter the cell cycle and arrested in G2 with RO-3306. In line with results from unperturbed cells (Figs. 3D and 5D), depletion of βTrCP or Plk1 or treatment with BI2536 stabilized USP37 in G2-arrested T98G cells (Fig. 6A). We next determined that phosphorylation of Ser-858 is required for destruction in G2. USP37 or the S858A mutant was expressed during the second thymidine block of HeLa Tet-On cells. Expression was shut off by releasing cells into doxycycline-free media, and protein stability was monitored in cells arrested in G2, as above. Consistent with our in vitro data, the S858A mutant remained stable, whereas the wild type protein was destroyed. Together we interpret these results to confirm that Plk1 and SCFβTrCP cooperate to trigger destruction of USP37 in G2.

To determine the physiological requirement for destruction of this pool of USP37, we determined the cell cycle profile of asynchronous HeLa cells that were transiently expressing USP37 or USP37-S858A by flow cytometry. Expression of USP37 caused a modest increase in the G2/M population, whereas expression of βTrCP-resistant USP37 caused a 2-fold increase of the G2/M population (Fig. 6, C and D). Immunofluorescence and flow cytometry analyses revealed increased cyclin B1-positive, nonmitotic cells in USP37 S858A-expressing cells, but not the mitotic markers MPM-2 and phospho-histone H3 (Ser-10), consistent with an accumulation in G2 rather than mitosis (Fig. 6, E–H). Taken together, we conclude that failure to degrade USP37 during G2 prevents mitotic entry.

**DISCUSSION**

In this study, we have demonstrated that the deubiquitinating enzyme USP37 undergoes biphasic degradation during late G2 and mitotic exit/early G1. We confirmed that USP37 is targeted for destruction at mitotic exit and in G1 by the APCCdh1 ligase as indicated by our previous study (22). We have presented several lines of evidence implicating SCFβTrCP and Plk1 as the ligase and triggering kinase responsible for USP37 destruction in G2: (i) USP37 interacts with Plk1, βTrCP, and components of the SCF in vitro and in vivo; (ii) expression of βTrCP or Plk1 down-regulates USP37 in a proteasome-dependent manner, whereas dominant-negative proteins increase USP37 levels; (iii) βTrCP induces USP37 ubiquitination in vitro and in vivo; (iv) phosphorylation of USP37 by Plk1 promotes binding to βTrCP and components of the SCF; (v) loss of βTrCP or Plk1 activity by siRNA or chemical inhibition stabilizes USP37; and (vi) mutation of the βTrCP degron stabilizes USP37. Together these data indicate that phosphorylation by Plk1 leads to SCFβTrCP-mediated ubiquitination and subsequent destruction of a pool of USP37 during G2.

Our data identify USP37 as a node in the complex circuitry connecting the APC and SCF ligases throughout the cell cycle. USP37 joins Cdc25A and Claspin as an S-phase regulator that is coordinately regulated by SCFβTrCP and APCCdh1 (10, 12, 18, 19, 37). A significant portion of USP37 remains throughout mitosis. These observations suggest an improved model for the biological role of USP37. Inhibition of APCCdh1 promotes...
S-phase entry. USP37 then bifurcates into two pools, one of which controls substrates that prevent mitotic entry. Destruction of this pool by SCF\(^{TrCP}\)/H9252TrCP promotes the G2/M transition. We postulate that the remaining mitotic-stable pool promotes mitotic progression and must be degraded to promote the G1 state, similar to many APCCdh1 substrates. Further studies will be required to define the roles of these pools and how the mitotic pool remains stable. Certainly, identification of additional USP37 substrates will be a major step in elucidating the function of these pools of USP37.

In contrast to USP37, βTrCP-resistant mutants of the APC inhibitor Emi1 cause accumulation of APC substrates, including cyclin A, which must also be degraded for progression past prometaphase (13, 42, 43). Although USP37 is implicated in its stability, cyclin A is unlikely to mediate the cell cycle effects we have observed (22). First, failure to degrade cyclin A results in delay in mitosis rather than G2 (42, 43). Second, cyclin A levels are not altered when USP37 levels drop in G2-arrested cells. Although we cannot exclude that cyclin A stability is mediated by the remaining pool of USP37, this is unlikely to be the case as cyclin A is degraded in mitosis despite the presence of this fraction of USP37. This is also consistent with the specificity of USP37 for APCCdh1 and the dependence of cyclin A destruction in G2 and M upon APCCdc20 (22, 44). These data suggest that
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destruction of USP37 in G2 is not prerequisite for destruction of cyclin A and indicate additional substrates. Intriguingly, the distinct cell cycle arrests caused by βTrCP-resistant USP37 and Em1 also suggest that these inhibitors regulate specific population of APC/Cdh1.

In summary, the results of our study further confirm the role of USP37 as a potent cell cycle regulator and underscore the need for identifying additional substrates of this enzyme to gain a better understanding of its critical functions.

Acknowledgments—We thank Xiaodong Huang and Vishva Dixit for the USP37 antibody; Janet Houghton and Tapati Mazumdar for assistance with flow cytometry; and Dipali Date, Haiyong Yang, Saurav Misra, and Monica Venere for helpful discussions and critical reading of the manuscript.

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