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Anaphase-Promoting Complex/Cyclosome Participates in the Acute Response to Protein-Damaging Stress[▽]

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The ubiquitin E3 ligase anaphase-promoting complex/cyclosome (APC/C) drives degradation of cell cycle regulators in cycling cells by associating with the coactivators Cdc20 and Cdh1. Although a plethora of APC/C substrates have been identified, only a few transcriptional regulators are described as direct targets of APC/C-dependent ubiquitination. Here we show that APC/C, through substrate recognition by both Cdc20 and Cdh1, mediates ubiquitination and degradation of heat shock factor 2 (HSF2), a transcription factor that binds to the *Hsp70* promoter. The interaction between HSF2 and the APC/C subunit Cdc27 and coactivator Cdc20 is enhanced by moderate heat stress, and the degradation of HSF2 is induced during the acute phase of the heat shock response, leading to clearance of HSF2 from the *Hsp70* promoter. Remarkably, Cdc20 and the proteasome 20S core α2 subunit are recruited to the *Hsp70* promoter in a heat shock-inducible manner. Moreover, the heat shock-induced expression of *Hsp70* is increased when Cdc20 is silenced by a specific small interfering RNA (siRNA). Our results provide the first evidence for participation of APC/C in the acute response to protein-damaging stress.

Strict regulation of gene expression is fundamental for normal growth and development. This regulation involves a sophisticated interplay between transcription factors present at the right place, at the right time, and in the appropriate amounts. For this purpose, cells make use of the ubiquitin-proteasome pathway to control the fast turnover of specific transcription factors (65). In the ubiquitin-proteasome pathway, the destruction of proteins is carried out by the 26S proteasome, consisting of a 20S core of α and β subunits with protease activity, flanked by the 19S regulatory complexes, which possess ATPase- and substrate-recognizing properties (64, 74). The protein destined for degradation is posttranslationally modified by a polyubiquitin chain that is recognized by the 19S lid (74). Conjugation of ubiquitin to the target requires an enzymatic cascade, consisting of an E1-activating enzyme, an E2-conjugating enzyme, and an E3 ligase that recognizes both E2 and the substrate, leading to the final attachment of ubiquitin (16).

The E3 ligases are crucial for determining the specificity of substrate ubiquitination, and many of them can be divided into two mechanistically different groups based on whether they contain a HECT (homologous to E6-AP carboxyl terminal) or a RING (really interesting new gene) domain (79). The anaphase-promoting complex/cyclosome (APC/C) is a RING E3 ligase responsible for proteolytic destruction during mitosis and is required for unidirectionality of the cell cycle (66). The mammalian APC/C consists of at least a dozen subunits, of which APC2 and APC3/Cdc27 are fundamental in the ubiquitin ligation reaction. Additionally, two WD40 domain-containing coactivators, Cdc20 and Cdh1, are needed for substrate recognition, and they interact with APC/C at different phases of the cell cycle, thereby contributing to the selectivity in substrate recognition (70). APC/C^{Cdc20} is active during mitosis, where it controls the precise timing of sister chromatid separation through degradation of cyclin B and the separase inhibitor securin (27, 34, 48, 88, 105). In late mitosis, the coactivator Cdc20 is replaced by Cdh1, which is required for exit from mitosis, and APC/C^{Cdh1} remains active during G_1 and early S phases (23, 52). The activation capacity of Cdc20 is regulated by its abundance and by inhibitor molecules, such as Mad2 and BubR1 (22, 44, 87, 89, 90). Cdc20 is downregulated in late mitosis by APC/C^{Cdh1}, and in S phase, the amount of Cdc20 begins to increase again (23, 71, 77, 96, 102).

Proteolysis is inherently associated with inhibition of protein activities, which also applies to a number of cases of APC/C-mediated degradation, when the substrate protein is either an enzyme or its cofactor. The repertoire of APC/C substrates includes mitosis-specific kinases, e.g., Aurora kinases and Polo-like kinase 1 (Plk1), and mitotic A- and B-type cyclins, the activating subunits of cyclin-dependent kinases 1 and 2 (Cdk1 and Cdk2) (18, 31, 56). The degradation of these substrates leads to loss of their kinase activities at specific time points of mitosis, consequently driving the cell cycle forward. On the other hand, APC/C can activate certain proteins, e.g., separase, by targeting their inhibitors, e.g., securin, for destruction (70). To date, only a few transcription factors have been identified as APC/C substrates. For example, FoxM1, AML1/RUNX1, and HOXC10 are targeted by APC/C for degradation in a cell cycle-dependent manner (12, 28, 69).

The heat shock response promotes cell survival in response

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to protein-damaging stressors, including elevated temperature, heavy metals, and bacterial and viral infections. The rescue is mediated by an increased expression of heat shock proteins (Hsps) that function as molecular chaperones maintaining protein homeostasis (62). The inducible expression of Hsps, e.g., Hsp70, depends on HSF1, which belongs to the heat shock transcription factor family, consisting of four members, HSF1 to HSF4 (6). The activity of HSF1 is regulated by several posttranslational modifications, such as phosphorylation, sumoylation, and acetylation (32, 37, 38, 40, 49, 83, 97). The stress-induced activation cycle of HSF1 is a multistep process that involves trimerization for DNA-binding activity, hyperphosphorylation for maximal transactivation, and attenuation of DNA-binding and transcriptional activities. In mammalian cells, the DNA binding of HSF1 occurs within minutes upon exposure to moderate heat stress at 42°C. Under these conditions, the transcription of *Hsp70* is acutely induced, reaching maximal levels within 1 h, followed by gradual attenuation (1, 49, 83, 85, 86). Inactive HSF1 is monomeric, and when activated, it forms either homotrimers or heterotrimers with HSF2. The trimers recognize specific DNA sequences, called heat shock elements (HSEs), within the target gene promoters (9, 82). In contrast, HSF2 exists predominantly as dimers in unstressed cells (86), where some constitutive HSF2 DNA-binding activity can also be detected (68). Both genetic and biochemical studies demonstrate that HSF1 is absolutely required for stress-inducible induction of Hsps (3, 4, 19, 41, 61, 76). However, recent studies have revealed that HSF2 also contributes to the heat shock response in collaboration with HSF1 (36, 58, 68, 82). For instance, during heat shock, HSF2 colocalizes with HSF1 in nuclear stress bodies (nSBs), where they regulate the expression of noncoding *Satellite III* (*Sat III*) RNA transcripts primarily from a heterochromatic locus on human chromosome 9 (7, 43). The formation of nSBs is considered to be a marker for activated heat shock response, although the function of the *Sat III* transcripts is still unclear (11). In addition to the *Hsp* promoters and *Sat III* DNA, HSF2 has been shown to occupy specific promoters during developmental and differentiation-related processes, such as *p35* in corticogenesis and Y-chromosomal multicopy genes in spermatogenesis (5, 14). Interestingly, HSF2 has also been demonstrated to mediate bookmarking of the inducible *Hsp70* gene (99).

Similarly to many short-lived transcription factors that influence gene expression in a temporal manner, the amounts of HSF2 vary between different tissues and in cells exposed to stressors such as proteasome inhibition and heat shock (14, 25, 59, 78, 82). It is unclear by which mechanism distinct HSF2 levels are accomplished under various conditions. Moreover, it remains to be shown how the stress-induced fluctuation of HSF2 levels affects the dynamics of HSF2 binding to its target promoters. Here we demonstrate that during the acute phase of heat stress, HSF2 is rapidly subjected to degradation by the ubiquitin-proteasome pathway, whereas HSF1 is more stable and resistant to ubiquitination. We found that APC/C mediates the stress-inducible ubiquitination of HSF2. Specifically, coimmunoprecipitation (co-IP) analyses showed that HSF2 interacts with the APC/C coactivators Cdc20 and Cdh1 in addition to the Cdc27 subunit. Furthermore, chromatin immunoprecipitation (ChIP) experiments revealed that Cdc20 occupies the *Hsp70* promoter in response to heat shock. Cdc20 availability has functional consequences in heat-stressed cells, since the expression of both *Hsp70* and *Sat III* was disturbed by specific small interfering RNA (siRNA)-mediated silencing of Cdc20. Because the proteasome subunit α2 was also recruited to the *Hsp70* promoter, we propose that the acute response to proteotoxic stress is delicately modulated by adjusting the abundance of promoter-bound HSF2. This spatiotemporal regulation is facilitated by recruitment of Cdc20 to the *Hsp70* promoter, suggesting that APC/C actively participates in the heat shock response.

MATERIALS AND METHODS

Plasmids. The plasmids encoding Myc-tagged ubiquitin, lysine mutants of Myc-ubiquitin, Myc-tagged HSF1 and HSF2, untagged and Flag-tagged HSF2, Cdc20-green fluorescent protein (GFP), and Myc-tagged Cdc20 and Cdh1 have been described earlier (3, 7, 38, 39, 44, 63, 72, 76). Glutathione *S*-transferase (GST)–ubiquitin was a kind gift from Jorma Palvimo (University of Eastern Finland, Kuopio, Finland).

Cell culture, transient transfections, and experimental treatments. Human embryonic kidney HEK293 and cervical cancer HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin, and streptomycin. Human K562 erythroleukemia cells were maintained in RPMI 1640 medium with the above-mentioned supplements. All cell types were maintained at 37°C in a humidified 5% CO_2 atmosphere. For transfections, 5×10^6 HEK293 cells were resuspended in 400 μl Opti-MEM (Gibco) together with 10 to 30 μg DNA and transferred to BTX cuvettes. The cells were electroporated (220 V, 975 μF) using a Bio-Rad gene pulser. After transfection, the cells were left to recover for 24 h before further treatment. For siRNA transfections, scrambled siRNA (1027289, AllStars negative control; Qiagen) and mixtures of specific oligonucleotides targeting human Cdc20, Cdh1, Cdc27, or APC2 (GeneSolution siRNA; Qiagen) were purchased and transfected in 1.5×10^5 HEK293 or HeLa cells by using RNAiMAX transfection reagent (Invitrogen). The transfected cells were left to recover for 48 h before treatment and harvesting. Heat shock treatments were performed in a 42°C water bath for the times indicated in the figures. MG132 (Peptide Institute Inc.) was used at a concentration of 20 μM for 5 h. Cycloheximide (Sigma) was used at a concentration of 10 μg/ml for the times indicated in the figures. Nocodazole-arrested HeLa cells were obtained by treating cells with 2 mM thymidine (Sigma) for 24 h, after which the thymidine was removed by washing. The cells were cultured for 6 h before addition of 100 ng/ml nocodazole (Fluka) and further incubated for 6 h. The mitotic cells were collected by mitotic shakeoff and harvested.

ChIP. The chromatin immunoprecipitation (ChIP) assay was performed as previously described (68). Briefly, 5.5×10^7 K562 cells or a 10-ml plate of HeLa cells was heat shocked for the times indicated in the figures and immediately cross-linked with a final concentration of 0.4% formaldehyde, followed by quenching with glycine in a final concentration of 125 mM. After harvest, the cells were lysed and DNA was fragmented by sonication with a Bioruptor system (Diagenode), followed by preclearing of the immunoprecipitation (IP) samples with protein G-Sepharose beads (GE Healthcare) at 4°C. The IP was performed at 4°C overnight with the following antibodies: anti-HSF1 (SPA-901; Stressgen), anti-HSF2 SFI58 (68), anti-Cdc20 (A301-179A; Bethyl Laboratories, Inc.), anti-α2 (PW8105; Biomol International, Inc.), and normal rabbit serum (Jackson ImmunoResearch Laboratories) as a nonspecific (NS) antibody. After extensive washing of the immunocomplexes, cross-links were reversed by incubation overnight at 65°C, and then DNA was purified with phenol-chloroform. PCR analysis was performed by using pure *Taq* Ready-to-go PCR beads (GE Healthcare) and primers for human *Hsp70.1* and β-*actin* (68). The input lanes represent 1% of the material used in the IP assay. For real-time PCR-based quantitative ChIP, samples were prepared as described above. Quantitative real-time PCR analysis was performed using an ABI Prism 7900HT system (Applied Biosystems), Absolute SYBR green ROX mix (Thermo Scientific), and primers for the human *Hsp70* promoter (hHsp70.1F, 5′-CTG GCC TCT GAT TGG TCC AA-3′; hHsp70.1R, 5′-CAC GGA GAC CCG CCT TTT-3′). IP samples were normalized to values obtained for input before fold enrichment was determined. The statistical analysis was performed by using paired two-tailed Student's *t* test.

IP and Western blotting. The co-IP studies were performed on transfected HEK293 cells as described previously (37). The following antibodies were used for IP: anti-Cdc20 (A301-179A; Bethyl Laboratories, Inc.), anti-Cdc27 (ab10538;

AbCam), and anti-Cdh1 (MS-1116; Thermo Scientific). The IP and input samples were run on an SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Protran nitrocellulose; Schleicher & Schuell). For total cell lysates, the treated or untreated cells were lysed directly in Laemmli sample buffer and subjected to SDS-PAGE. Antibodies used for immunoblotting were anti-HSF1 (39) and anti-HSF2 SFI58, anti-GFP (JL-8; Clontech), anti-Cdc27 (610454; BD Biosciences), anti-Cdc20 (A301-180A; Bethyl Laboratories, Inc.), antiubiquitin FK2 (PW8810; Biomol International, Inc.), anti-Cdh1 (MS-1116; Thermo Scientific), anti-Myc (2276; Cell Signaling Technology), anti-APC2 (550362; BD Pharmingen), antitubulin (T8203; Sigma), and anti-Hsc70 (SPA-815; Stressgen). Secondary antibodies conjugated to horseradish peroxidase were purchased from Promega, GE Healthcare, and Southern Biotechnology Associates, Inc., and immunocomplexes were detected by enhanced chemiluminescence (ECL; GE Healthcare). The densitometry was performed using ImageJ (NIH), and the values for HSF2 were normalized to those for Hsc70 or tubulin.

Denaturing ubiquitination assay. Untransfected and transfected HEK293 cells were harvested, suspended in 20 μl cold phosphate-buffered saline (PBS), and lysed in 100 μl boiling 1% SDS-PBS. The samples were boiled for 8 min prior to addition of 650 μl of 1% Triton X-100–PBS. To break DNA, the samples were homogenized by several strokes with a needle, followed by a short centrifugation to enable collection of 30 μl of the supernatant as the input sample. Before centrifugation at 15,000 × *g* for 15 min at 4°C, 650 μl of 1% Triton X-100–1% bovine serum albumin (BSA)–PBS was added to the rest of the sample. The supernatant was incubated with 5 μl of anti-HSF1 (SPA-901; Stressgen) or anti-HSF2 SFI58 antibody together with 20 μl of 50% protein G Sepharose slurry for 2 h at 4°C. The beads were washed 4 times with 1 ml 1% Triton X-100–PBS, followed by boiling in Laemmli sample buffer. The IP and input samples were analyzed by SDS-PAGE and immunoblotting.

***In vitro* ubiquitination and pulldown assays.** The *in vitro* ubiquitination assay was performed as described previously (51). In short, untreated, heat-shocked (30 min at 42°C), nocodazole-arrested HeLa cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 20 mM β-glycerophosphate, 5 mM $MgCl_2$, 0.2% NP-40, 10% glycerol, 1 mM NaF, 0.5 mM dithiothreitol [DTT], and protease inhibitor cocktail [Roche]). APC/C was immunopurified from the lysates with anti-Cdc27 antibody (clone AF3.1; Sigma) coupled to Affiprep protein A beads (Bio-Rad). APC/C-linked beads were added to an *in vitro* reaction mixture containing UBE1 as E1 and UbcH10 as E2 (Boston Biochem), GST-ubiquitin, an ATP regenerating system, and *in vitro*-translated HSF2 (Promega). The reaction mixture was incubated at 37°C for 45 min, and the samples were analyzed by SDS-PAGE and immunoblotting with anti-HSF2 antibody.

The *in vitro* pulldown assay was performed as previously described (72), with minor modifications. Briefly, *in vitro*-translated Myc-tagged empty vector, Cdc20, or Cdh1 was preincubated with Myc-agarose beads (Sigma) in binding buffer (50 mM HEPES, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, and 0.2% Tween 20), followed by addition of *in vitro*-translated HSF2 that was ^{35}S-labeled with EasyTag EXPRESS^{35}S protein labeling mix (Perkin Elmer). After incubation for 2 h at 4°C, the beads were washed four times with binding buffer and boiled in Laemmli sample buffer, followed by analysis by SDS-PAGE and autoradiography.

Quantitative real-time reverse transcription-PCR (RT-PCR). RNA was isolated using an RNeasy kit (Qiagen). Contaminating genomic DNA was removed with two DNase I treatments according to the RNeasy protocol (Qiagen). Of each sample, 1 μg of RNA was subjected to reverse transcription using a high-capacity cDNA reverse transcription kit (Applied Biosystems). Kapa Probe Fast ABI Prism qPCR mix (Kapa Biosystems) was used for *Hsp70* analysis and Kapa SYBR Fast ABI Prism qPCR mix for *Sat III*. The PCRs were performed with an ABI Prism 7900HT system (Applied Biosystems). Relative quantities of *Hsp70* or *Sat III* transcripts were normalized against their respective *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase gene) transcripts, and fold inductions were determined. The results were analyzed with SDS 2.3 and RQ Manager software (Applied Biosystems). Primers and probes for *Hsp70* and *GAPDH* have been described previously (68), as have primers for *Sat III* and *GAPDH* (82). The statistical analysis was performed by using paired two-tailed Student's *t* test.

RESULTS

Transient DNA-binding activity and rapid turnover of HSF2 in response to acute heat stress. For maintaining protein homeostasis, the immediate response to proteotoxic stress is under strict control of the heat shock response pathway. Although HSF1 is the master regulator of the heat shock response (61), recent studies demonstrate that HSF2 also participates in the stress-inducible activation of *Hsp* transcription (36, 68, 82). We have shown previously that upon 1 h of heat shock at 42°C, HSF2 is recruited to the *Hsp70* promoter in an HSF1-dependent manner (68). To examine the dynamics of HSF1 and HSF2 occupancy at the *Hsp70* promoter, we subjected human K562 cells to a heat shock time course at 42°C and performed quantitative chromatin immunoprecipitation with anti-HSF1 and anti-HSF2 antibodies. The maximal promoter binding of both HSF1 and HSF2 was detected as rapidly as at 15 min of heat shock (Fig. 1A). Unlike HSF1, which stayed at the promoter throughout the time course up to 45 min, HSF2 occupancy had already started to decline after 30 min of heat shock (Fig. 1A).

The reduction in the amount of promoter-bound HSF2 could be due to a decline in the protein level of HSF2. To explore the consequences of elevated temperature on HSF2 concentration, we monitored the HSF2 protein levels in human K562 and HEK293 cells exposed to moderate heat stress at 42°C. The amount of HSF2 started to decrease after a 30-min treatment and was clearly reduced at 60 min (Fig. 1B and data not shown). Since the function of many transcription factors is regulated by their turnover (35, 47, 57, 60, 81), we next investigated the stability of HSF2 in the absence of heat stress by treating cells with the protein synthesis inhibitor cycloheximide. As shown in Fig. 1C, HSF2 had a short half-life of approximately 1 to 2 h, whereas the amount of HSF1 was not markedly decreased even after 6 h of cycloheximide treatment.

Ubiquitination of HSF2 is rapidly enhanced during heat shock. Proteins with short half-lives are often degraded by the ubiquitin-proteasome pathway (16, 17, 24, 73). To examine whether HSF1 and HSF2 are targeted for ubiquitination, we performed a ubiquitination assay in HEK293 cells treated with the proteasome inhibitor MG132. The immunoprecipitation was performed with antibody recognizing HSF1 or HSF2, followed by detection of endogenously ubiquitinated proteins with antiubiquitin antibody. Ubiquitinated HSF2 was also present in untreated cells but was markedly increased in response to proteasome inhibition (Fig. 2A). In contrast, the amount of ubiquitinated HSF1 in MG132-treated cells was almost at the background level compared to that for the no-antibody control (Fig. 2A, compare lanes 2 and 5), indicating only a minor ubiquitination of HSF1. Treatment with MG132 or other proteasome inhibitors leads to accumulation of misfolded and nondegraded proteins and, consequently, induction of the heat shock response (39, 45, 59, 76). Upon activation, HSF1 is hyperphosphorylated, which is observed by the retarded mobility of HSF1 on SDS-polyacrylamide gels (Fig. 2A, lane 2). The results presented in Fig. 1 and 2A suggest that HSF2 is an unstable protein that is modified prominently by conjugation of ubiquitin.

Because the heat shock-induced transcription of *Hsp70* reaches the maximal level before 1 h of treatment at 42°C (1), by which time a decrease in HSF2 protein and promoter occupancy was already observed (Fig. 1A and B), we subjected cells to a heat shock time course up to 1 h and monitored the kinetics of ubiquitin conjugation to HSF2. Analysis of the time course revealed that ubiquitination of HSF2 was enhanced, peaking rapidly at 30 to 40 min of heat shock (Fig. 2B), followed by a decline by 60 min of heat treatment. Possibly, by

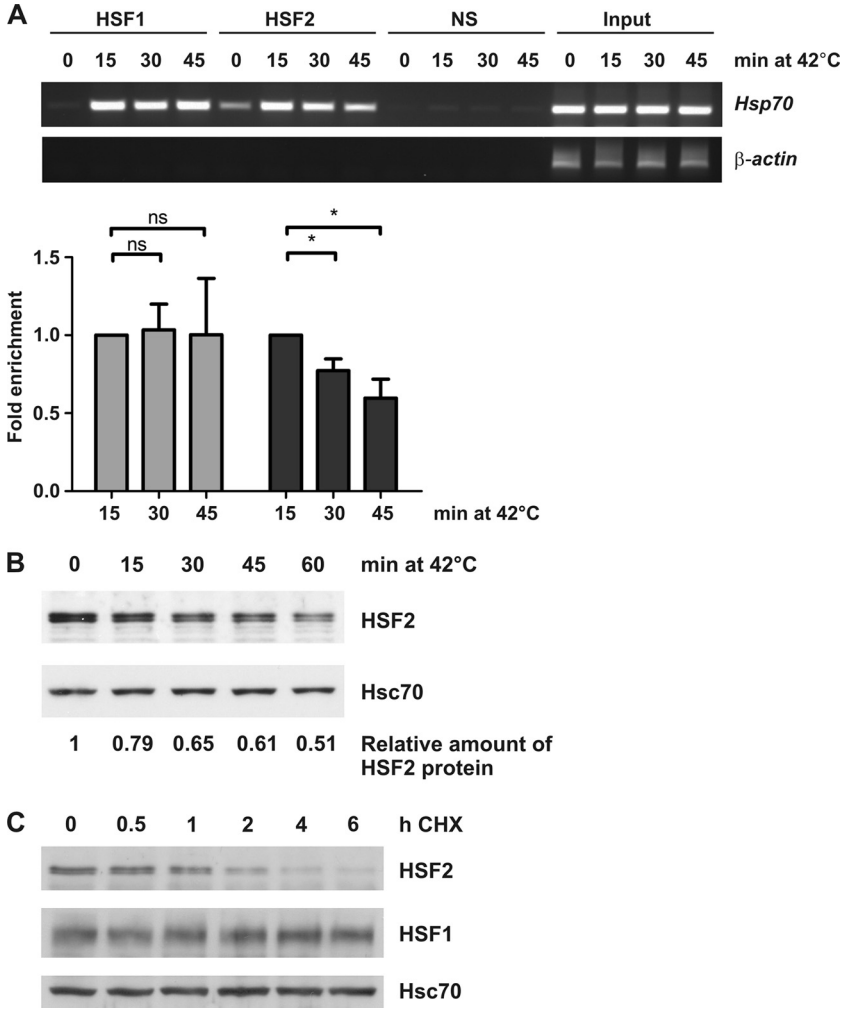

FIG. 1. HSF2 DNA-binding activity and protein levels are decreased during heat shock. (A) ChIP was performed on K562 cells heat shocked at 42°C for the indicated times, and the samples were immunoprecipitated with anti-HSF1, anti-HSF2, or nonspecific (NS) antibody. The IP and input samples were subjected to PCR using primers specific for the human *Hsp70* and β-*actin* promoters. The promoter occupancies of HSF1 and HSF2 were statistically analyzed by performing paired two-tailed Student's *t* test on values obtained by quantitative real-time PCR on ChIP samples. The maximal binding for both HSF1 (light bars) and HSF2 (dark bars) was detected upon 15 min of heat shock and was set to 1. Error bars represent standard errors of the means from four independent experiments. ns, no significance; *, $P < 0.05$. (B) HEK293 cells were treated with heat shock at 42°C for the indicated times, and total Laemmli lysates were run on SDS-polyacrylamide gels. The protein levels of HSF2 were detected by blotting with anti-HSF2 antibody, and equal loading was confirmed by blotting with anti-Hsc70 antibody. The relative amount of HSF2 protein (indicated below the blot) was determined by densitometry and normalized to that of Hsc70. The highest HSF2 level was detected at the 0-min time point and was set to 1. (C) HEK293 cells were treated with cycloheximide (CHX) (10 μg/ml) at 37°C for the indicated times, and total Laemmli lysates were analyzed by blotting with anti-HSF1 or anti-HSF2; anti-Hsc70 antibody was used to confirm equal loading.

this time point, the amount of HSF2 protein had decreased to a level where less HSF2 was available for ubiquitination. The dynamics in HSF2 ubiquitination, with an induction within 30 min of treatment, followed by a subtle decline with longer heat shock, implies a strict regulation of the availability of HSF2 during the acute phase of the heat shock response.

The ubiquitin chain formed on a target protein can be assembled through different lysine residues on the ubiquitin molecule, such as K6, K11, K27, K29/33, K48, or K63 (2, 10, 15, 20, 21, 30, 67, 95). Depending on the type of chain assembled, the ubiquitination modification assigns specific outcomes for the target protein. The K48 and K11 chains typically lead to recognition by the proteasome and subsequent degradation, whereas chains assembled through K63 have nonproteolytic functions in DNA damage repair, cellular signaling, intracellular trafficking, and ribosomal biogenesis (75, 101). To examine whether the ubiquitin chain forming on HSF2 indeed leads to degradation, we expressed in HEK293 cells K11R, K48R, and K63R mutants of Myc-tagged ubiquitin and analyzed the ubiquitination of HSF2 upon heat shock. HSF2 was ubiquitinated to a lesser extent in the presence of the K11R or the K48R mutant than in the presence of wild-type ubiquitin, whereas in cells expressing the K63R mutant, HSF2 was ubiquitinated similarly to the level seen with the wild type (Fig. 2C). This result indicates that the ubiquitin chains forming on HSF2 upon heat shock consist of K11-linked, K48-linked, or perhaps mixed ubiquitin moieties, suggesting that the consequence of HSF2 ubiquitination is degradation. This conclusion is sup-

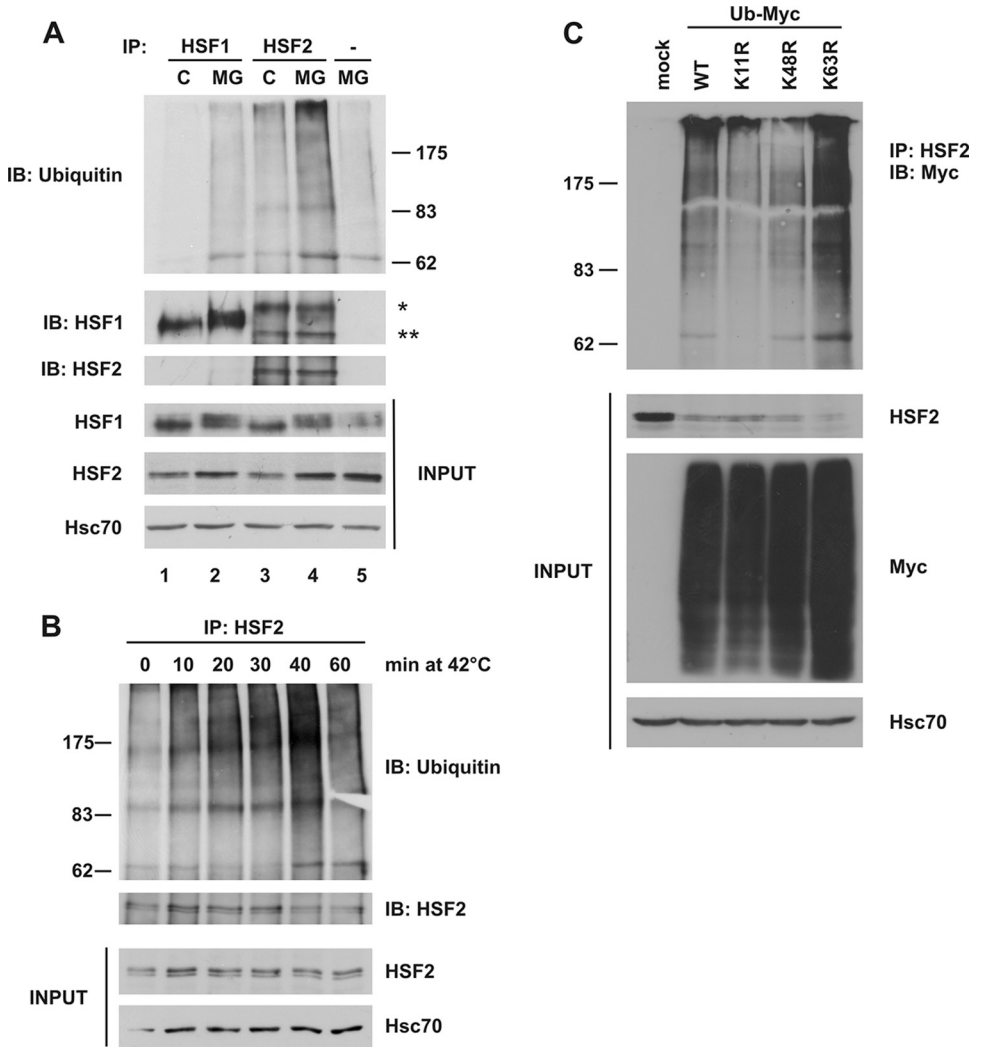

FIG. 2. Ubiquitination of HSF2 is enhanced by heat stress. (A) HEK293 cells were left untreated (C) or treated with 20 μM MG132 (MG) for 5 h. IP was performed with anti-HSF1, anti-HSF2, or no antibody (−), and samples were run on SDS-polyacrylamide gels. Ubiquitinated proteins were subsequently detected with antiubiquitin antibody. The IP membrane was reblotted first with anti-HSF2 antibody and then with anti-HSF1 antibody, and therefore, HSF2 is also seen in the HSF1 blot (**). *, unspecific band. The inputs were blotted with anti-HSF1, anti-HSF2, and anti-Hsc70 antibodies. (B) HEK293 cells were treated with heat shock at 42°C for the indicated times. HSF2 was immunoprecipitated with anti-HSF2 antibody, and the samples were analyzed by SDS-PAGE followed by blotting with antiubiquitin antibody. Input samples were analyzed by immunoblotting (IB) with anti-HSF2 and anti-Hsc70 antibodies. (C) HEK293 cells were transfected with empty vector (mock) or Myc-tagged wild-type (WT) or mutant ubiquitin (Ub) where lysines 11, 48, and 63 were mutated to arginine (K11R, K48R, and K63R, respectively). The transfected cells were heat shocked for 30 min at 42°C. Anti-HSF2 antibody was used for IP, and ubiquitination was detected with anti-Myc antibody. The protein amounts in the input samples were detected with antibodies against HSF2, Myc, and Hsc70. The numerical values outside the blots indicate molecular size markers in kilodaltons.

ported by the increased ubiquitination of HSF2 in the presence of a proteasome inhibitor (Fig. 2A).

HSF2 interacts with APC/C coactivators Cdc20 and Cdh1. The target selectivity in the ubiquitin-proteasome pathway is provided by the E3 ligases, which specifically recognize target proteins (79). Further substrate specificity is provided by cofactors of E3 ligases, such as Cdc20 and Cdh1 for APC/C (70). HSF1 has been reported to interact with Cdc20 (54), and because HSF2 forms heterotrimers with HSF1, the E3 ligase activity of APC/C could be mediated to HSF2 by HSF1-HSF2 interaction. Furthermore, APC/C-stimulated ubiquitination has been shown to favor K11-linked ubiquitin chains (42, 98), which were also formed on HSF2 (Fig. 2C). In addition, APC/C is a nuclear E3 ligase, and HSF2 is localized in the nucleus upon heat shock (7, 70, 84). Based on these criteria, we explored the possibility of APC/C acting as an E3 ligase for HSF2. GFP-tagged Cdc20 was transiently overexpressed in HEK293 cells, and the protein levels of HSF2 were analyzed by immunoblotting. As shown in Fig. 3A, ectopic expression of Cdc20-GFP decreased the abundance of HSF2 to the same extent as heat shock (compare lane 1 with lanes 2 and 3).

To test whether HSF2 interacts with APC/C, we performed immunoprecipitation on endogenous Cdc20 and Cdc27, which is a core subunit of APC/C and operates in the ubiquitin

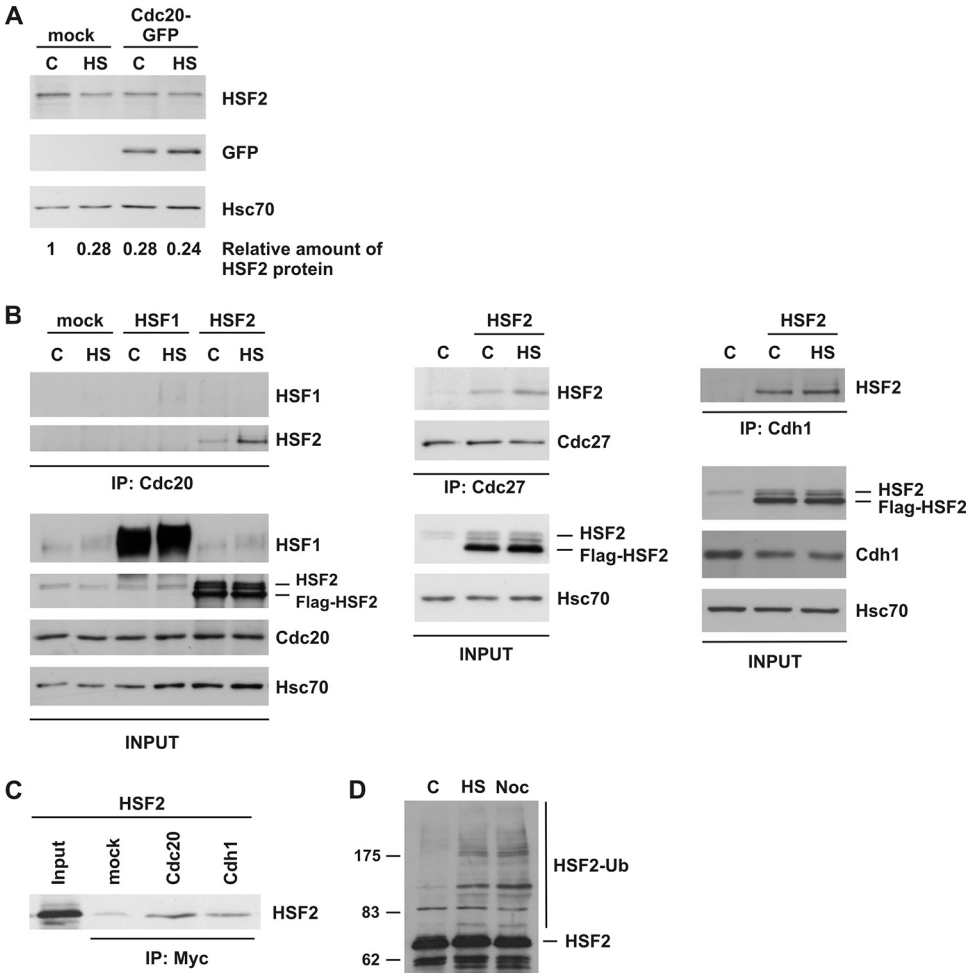

FIG. 3. HSF2 interacts with Cdc20, Cdh1, and Cdc27. (A) HEK293 cells were transfected with empty vector (mock) or Cdc20-GFP and left untreated (C) or heat shocked at 42°C for 30 min (HS). The cells were lysed, analyzed by SDS-PAGE, and immunoblotted with antibodies recognizing HSF2, GFP, and Hsc70. The relative amount of HSF2 protein (indicated below the blot) was determined by densitometry and normalized to that of Hsc70. The highest HSF2 level was detected in lane 1 and was set to 1. (B) (Left) HEK293 cells were transfected with empty vector (mock), HSF1-Myc, or Flag-HSF2, followed by no treatment (C) or heat shock at 42°C for 30 min (HS). IP was performed with anti-Cdc20 antibody and analyzed with anti-HSF1 or anti-HSF2 antibody. The input membranes were immunoblotted with antibodies recognizing HSF1, HSF2, Cdc20, and Hsc70. (Middle) Anti-Cdc27 IP was performed on HEK293 cells transfected with Flag-HSF2 and treated as described above. The IP samples were analyzed with anti-HSF2 and anti-Cdc27 antibodies, and the input samples were treated with anti-HSF2 and anti-Hsc70 antibodies. (Right) IP was performed with anti-Cdh1 antibody on cells transfected and treated as described above. Anti-HSF2, anti-Cdh1, and anti-Hsc70 antibodies were used for immunoblotting. (C) An *in vitro* pulldown assay was performed on reaction mixtures containing ^{35}S-labeled HSF2 and cold *in vitro*-translated Myc-tagged empty vector (mock), Cdc20, or Cdh1. Myc-tagged proteins were immunoprecipitated using Myc-agarose, and HSF2 was detected by autoradiography. The input sample contains 20% of the HSF2 volume used in the pulldown samples. (D) An *in vitro* ubiquitination assay was performed on *in vitro*-translated HSF2 as a substrate and immunopurified APC/C as an E3 ligase from HeLa cells that were untreated, heat shocked at 42°C for 30 min (HS), or arrested with nocodazole (Noc) (100 ng/ml) for 6 h. The reaction samples were incubated at 37°C for 45 min and run on SDS-polyacrylamide gels, followed by immunoblotting with anti-HSF2 antibody. Numbers on the left indicate molecular size markers in kilodaltons.

ligation reaction (91). Interestingly, immunoblotting with anti-HSF2 antibody revealed an interaction of Flag-tagged HSF2 and endogenous HSF2 with both Cdc20 and Cdc27 under control conditions (Fig. 3B, left and middle, and data not shown). The interaction was further enhanced in cells subjected to heat shock, implying that the stress-inducible ubiquitination of HSF2 could be mediated by the E3 ligase activity of APC/C. We also performed immunoprecipitation with Cdh1-recognizing antibody and found an interaction between this coactivator and HSF2 (Fig. 3B, right). However, the interaction between HSF2 and Cdh1 was not affected by heat shock treatment similarly to that between HSF2 and Cdc20 or Cdc27. To our surprise, no Cdc20-HSF1 interaction was detected using our model system (Fig. 3B, left), indicating an association between HSF2 and the APC/C complex without HSF1 being involved. Moreover, by use of *in vitro*-translated proteins, HSF2 was pulled down with Cdc20 and Cdh1, suggesting a direct interaction of Cdc20 or Cdh1 with HSF2 (Fig. 3C). Taken together, these results demonstrate that both Cdc20 and Cdh1 can associate with HSF2, connecting it to APC/C.

To study whether APC/C regulates the ubiquitination of HSF2, we performed an *in vitro* ubiquitination assay. For this

purpose, we immunoprecipitated APC/C with anti-Cdc27 antibody from control HeLa cells or cells that were exposed to heat shock or treated with the microtubule-depolymerizing drug nocodazole. Nocodazole treatment leads to accumulation of cells to M phase, which were used as a positive control for active APC/C (51). Immunoprecipitated APC/C was added to an *in vitro* ubiquitination reaction mixture containing E1, E2, GST-ubiquitin, and *in vitro*-translated HSF2 as a substrate. The reaction mixture was incubated at 37°C, and the reaction was terminated after 45 min. APC/C isolated from heat-shocked and mitotic cells, but not control cells, had equal capacities to promote ubiquitination of HSF2 (Fig. 3D), suggesting that APC/C functions as a ubiquitin E3 ligase for HSF2.

Silencing of APC/C subunits results in less ubiquitination of HSF2. The impact of APC/C on HSF2 stability was assessed by silencing coactivators or subunits of APC/C. Cdc20 either alone or in combination with Cdh1 was downregulated in HEK293 cells by specific siRNAs, and the turnover of HSF2 upon cycloheximide treatment was examined. A slightly prolonged turnover of HSF2 was detected in Cdc20- or Cdh1-silenced cells compared with that detected in scrambled control cells at the 1.5- and 2-h time points of cycloheximide treatment (Fig. 4A, left, compare lanes 3 and 4 with lanes 7 and 8). However, HSF2 was clearly stabilized in cells where both Cdc20 and Cdh1 were depleted, as evident at the 1-h and 1.5-h time points (Fig. 4A, top right, compare lanes 1 to 3 with lanes 5 to 7). Similarly, the depletion of Cdc27 increased the half-life of HSF2 from approximately 1 h in scrambled control cells to 1.5 h in Cdc27 siRNA-transfected cells (Fig. 4A, bottom right, compare lanes 1 to 3 with lanes 5 to 7).

Next, we analyzed the ubiquitination of HSF2 in cells deficient in Cdc20, Cdh1, or the APC/C subunit Cdc27 or APC2 (Fig. 4B). The heat shock-induced ubiquitination of HSF2 was not affected in HEK293 cells transfected with either Cdc20- or Cdh1-specific siRNAs compared with that in scrambled control cells. However, a combined depletion of both coactivators resulted in reduced HSF2 ubiquitination in cells exposed to heat stress (Fig. 4B, left). In addition, we used siRNA oligonucleotides specific for Cdc27 or APC2, a scaffolding subunit of APC/C that has cullin-like properties and is essential for E3 ligase activity (103, 104). Silencing of either subunit resulted in impaired induction of HSF2 ubiquitination compared with that for the scrambled control (Fig. 4B, right). These results indicate that the disruption of APC/C, through silencing its specific functional subunits, counteracts the stress-induced ubiquitination of HSF2.

As seen in Fig. 1B, the levels of HSF2 protein were decreased during a time course of heat shock treatment. To explore how downregulation of Cdc20 and Cdh1 affects the stability of HSF2 during heat shock, we transfected HEK293 cells with scrambled siRNA or Cdc20-specific siRNA alone or together with Cdh1-specific siRNA. The cells were subjected to a heat shock time course, and the kinetics of HSF2 reduction was monitored. Compared with the results for the scrambled control, the heat stress-induced reduction in HSF2 levels was partly rescued by Cdc20 downregulation (Fig. 5). The rescue was even more pronounced by a simultaneous knockdown of Cdc20 and Cdh1 (Fig. 5). These results highlight the importance of intact APC/C in the regulation of HSF2 abundance, specifically in the acute response to heat stress.

Heat shock-induced recruitment of Cdc20 to the *Hsp70* promoter. Because the APC/C disruption led to an HSF2 that is more resistant to heat shock-induced degradation, we analyzed by ChIP the occupancy of HSF2 on the *Hsp70* promoter in heat-shocked HeLa cells when Cdc20 was silenced. The scrambled transfected cells showed increased HSF2 occupancy at 15 min and reduced occupancy at 45 min. In contrast, no similar dynamics of promoter binding was observed in Cdc20-depleted cells (Fig. 6A, left), where HSF2 binding to the *Hsp70* promoter remained constant. Interestingly, although Cdc20 knockdown resulted in a diminutive increase in HSF2 protein levels, as observed by immunoblotting (Fig. 6A, right), the binding of HSF2 to the *Hsp70* promoter was not enhanced by heat stress with kinetics similar to that for cells expressing Cdc20. Thus, Cdc20 silencing interferes with the dynamics of HSF2 occupancy at the *Hsp70* promoter.

Since Cdc20 depletion altered the heat shock-induced kinetics of HSF2 occupancy on the *Hsp70* promoter (Fig. 6A) and Cdc20 participated in HSF2 ubiquitination (Fig. 4B), we investigated whether Cdc20 was present at the *Hsp70* promoter. If ubiquitination occurs at the promoter, one would presume that the specific ubiquitin E3 ligase was also found at the promoter (65). We performed ChIP with anti-Cdc20 antibody to examine whether the APC/C coactivator involved in HSF2 ubiquitination was found at the *Hsp70* promoter. Intriguingly, Cdc20 was recruited to the *Hsp70* promoter in a heat shock-inducible fashion and was already detected at the 15-min time point (Fig. 6B). Unlike dynamic HSF2 binding to the *Hsp70* promoter, the association of Cdc20 to this promoter remained constant during the entire heat shock time course (compare Fig. 1A and 6A and B). The occupancy of the *Hsp70* promoter by both HSF2 and Cdc20 indicated that the colocalization could be functionally associated with the ubiquitination of HSF2 and its subsequent degradation by the proteasome. This hypothesis was further supported by the results obtained from ChIP analysis with an antibody against the α2 subunit of the proteasome 20S core complex, showing an enhanced occupancy of the *Hsp70* promoter by the proteasome in heat-shocked cells (Fig. 6C). Taken together, our experiments revealed a stress-induced assembly of subunits of both APC/C and the proteasome at the *Hsp70* promoter.

Depletion of Cdc20 modulates the heat shock-induced transcription of *Hsp70* and *Sat III*. The alteration in the dynamics of HSF2 occupancy at the *Hsp70* promoter in cells lacking Cdc20 implies consequences for transcriptional regulation. To explore the impact of Cdc20 on *Hsp70* transcription, we analyzed by quantitative real-time RT-PCR HeLa cells exposed to a 1-h heat shock and transfected with either scrambled control or Cdc20 siRNA. Interestingly, *Hsp70* mRNA increased in Cdc20-silenced cells compared with the level for the scrambled control, indicating that Cdc20 impacts the stress-inducible *Hsp70* transcription (Fig. 7A). The same result was also obtained with mouse embryonic fibroblasts downregulating Cdc20 (data not shown). To further investigate whether Cdc20 has a general role in the regulation of the heat shock response, we analyzed the *Sat III* transcripts, which are synthesized in an HSF-dependent manner. While HSF1 is essential for heat shock-inducible transcription of the *Sat III* repeats (43, 82, 93),

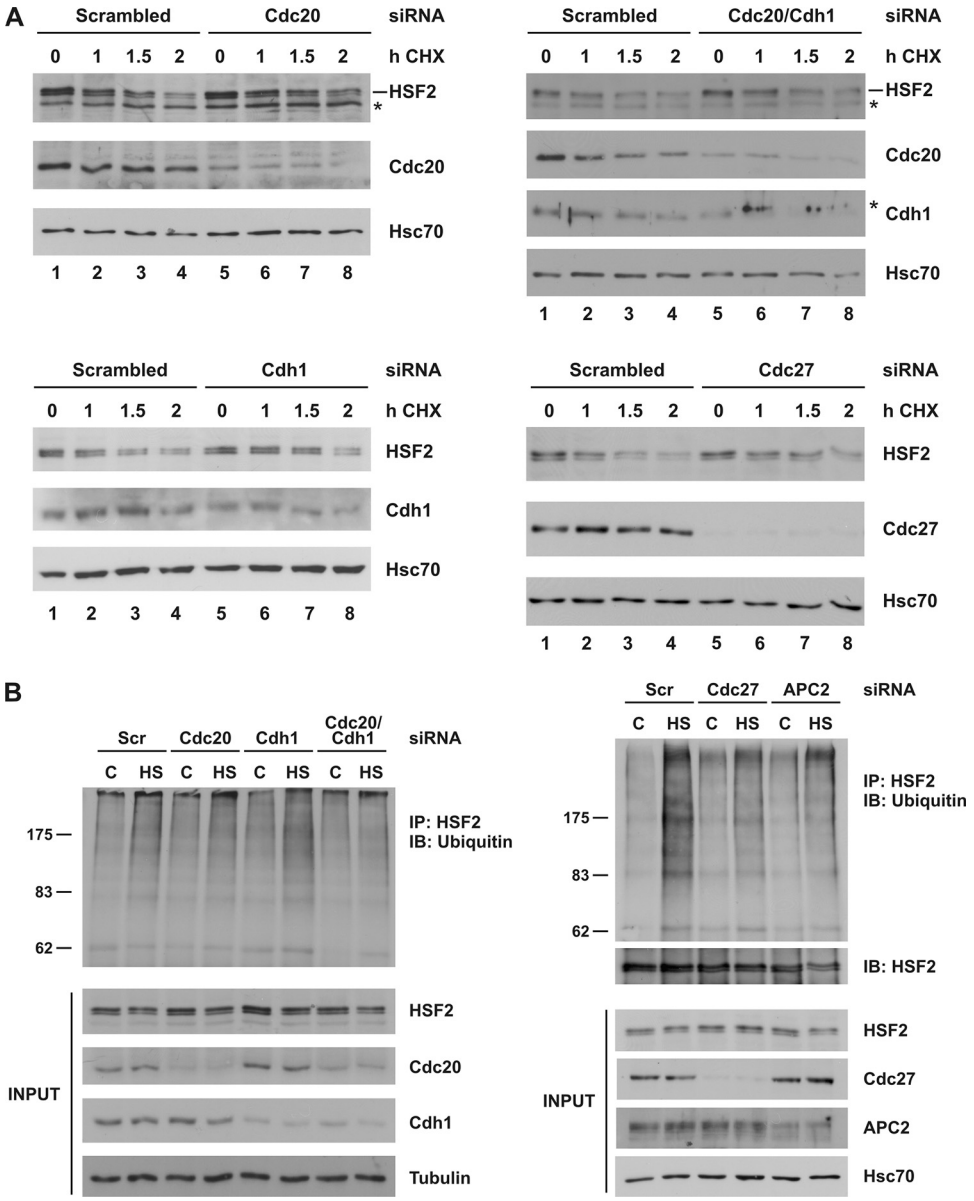

FIG. 4. Downregulation of APC/C subunits increases HSF2 stability. (A) Scrambled siRNA or siRNA oligonucleotides downregulating Cdc20 or Cdh1 (left), Cdc20 and Cdh1 (top right), or Cdc27 (bottom right) were transfected into HEK293 cells, followed by treatment with cycloheximide (CHX) (10 μg/ml). Laemmli lysates were harvested at the indicated time points and analyzed by SDS-PAGE. Protein levels of HSF2, Cdc20, Cdh1, Cdc27, and Hsc70 were detected by blotting with specific antibodies. *, unspecific band. (B) HEK293 cells were transfected with scrambled (Scr), Cdc20, and/or Cdh1 siRNA (left) or scrambled (Scr), Cdc27, or APC2 siRNA (right) and left untreated (C) or heat shocked for 30 min at 42°C (HS). The assay detecting HSF2 ubiquitination was performed as described in the legend for Fig. 2B. The input samples were blotted with anti-HSF2, anti-Cdc20, anti-Cdh1, anti-Cdc27, and anti-APC2 antibodies. Equal loading was controlled with antitubulin or anti-Hsc70 antibody. Numbers on the left indicate molecular size markers in kilodaltons.

elevated HSF2 levels have been reported to activate transcription in the absence of stress by recruiting HSF1 to the nSBs (82). The transcription of *Sat III* repeats is increased in heat-shocked HSF2-downregulated cells compared to that for the scrambled control, a result which is different from that for the *Hsp70* gene, whose expression is less induced in HSF2$^{-/-}$ cells than in their wild-type counterparts, indicating that HSF2 affects transcription in a target-dependent manner (68). Interestingly, silencing of Cdc20 led to diminished *Sat III* transcripts upon a 1-h heat shock treatment (Fig. 7B). These results imply that the APC/C coactivator Cdc20 regulates the transcription of heat shock-responsive genes, possibly by modifying the dynamics of HSF2 occupancy at the HSE-containing promoters in cells upon exposure to heat stress.

DISCUSSION

The activity and regulation of APC/C in the cell cycle have been studied extensively, and their importance in driving the ubiquitination of a multitude of regulatory proteins, such as

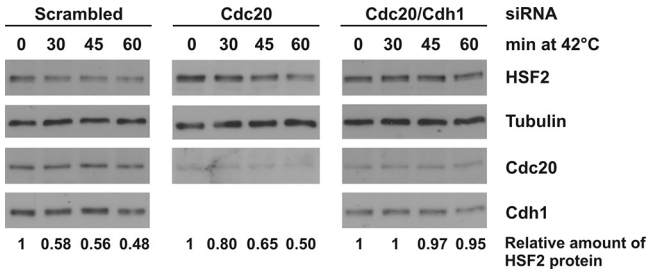

FIG. 5. HSF2 becomes more resistant to heat shock-induced degradation when APC/C coactivators are silenced. Laemmli lysates from HEK293 cells that were transfected with scrambled, Cdc20, or Cdc20 and Cdh1 siRNA and subjected to heat shock at 42°C for the indicated times were run on SDS-polyacrylamide gels. Immunoblotting was performed with anti-HSF2, antitubulin, anti-Cdc20, and anti-Cdh1 antibodies. The relative amount of HSF2 protein (indicated below the blots) was determined by densitometry and normalized to that of tubulin. The highest HSF2 level for each transfection was detected at the 0-min time point and was set to 1.

securin and mitotic cyclins, is well documented (70, 102). APC/C is a very stable complex whose functions are regulated via at least three E2 enzymes: UbcH5, UbcH10, and E2-25K (70, 80). Moreover, two WD40 domain proteins, Cdc20 and Cdh1, guide the APC/C activity toward specific substrates in a temporally controlled manner (102). Although the Cdc20/Cdh1 switch is particularly prominent in proliferating cells, both APC/C^{Cdc20} and APC/C^{Cdh1} have also been shown to function in nonproliferating postmitotic neurons, where APC/C is needed for regulation of dendrite morphogenesis, axonal growth, synaptic plasticity, and survival (8, 46, 50, 53, 94). A recent study by Yang and coworkers revealed that APC/C^{Cdc20} is crucial for presynaptic differentiation by triggering the degradation of transcription factor NeuroD2, leading to suppression of its target genes, such as *Complexin II* (100). Unexpectedly, we found evidence for a completely new role for APC/C in the heat shock response through a stress-inducible recruitment of Cdc20 to the *Hsp70* promoter.

The *Hsp70* promoter is inducibly bound and activated by HSF1 and HSF2 in response to heat stress. In this study, we discovered a direct association between HSF2 and the APC/C coactivators Cdc20 and Cdh1. The association seems specific for HSF2, since HSF1 was not found to interact with Cdc20. Interestingly, both the interaction of HSF2 with Cdc20 and ubiquitination were augmented by heat shock. A similar stress-inducible interaction was also detected with Cdc27, which is a tetratricopeptide repeat-containing APC/C core subunit that interlinks the coactivators to the APC/C core (91), indicating that APC/C promotes HSF2 ubiquitination. Furthermore, HSF2 was also found to coprecipitate with Cdh1. The finding that HSF2 associates with both Cdc20 and Cdh1 is not unique, since other substrates have also been shown to be regulated by both coactivators (12, 92) and some redundancy in substrate recognition occurs in the absence of either Cdc20 or Cdh1 (26, 29, 33, 55). Moreover, HSF2 exhibited a longer half-life and less heat shock-induced ubiquitination when APC/C coactivators or subunits were silenced by specific siRNAs. Similarly, the downregulation of HSF2 upon heat shock treatment was rescued in cells where the APC/C coactivators were depleted. Taken together, these results suggest that HSF2 is ubiquitinated in a heat shock-inducible fashion by APC/C, leading to destruction of HSF2 by the 26S proteasome.

The binding of HSF1 to the HSEs within the *Hsp70* promoter is instantaneous upon exposure to heat stress, and the transcriptional induction of *Hsp70* occurs rapidly and reaches the maximal level within the first hour of insult. The occupation of the *Hsp70* promoter and transcription are sustained with continuous stress until attenuation occurs and HSFs are released from DNA (1, 85, 97). We investigated more closely the dynamics of DNA-binding activity of HSF1 and HSF2 at the *Hsp70* promoter and noticed that the stress-inducible binding of HSF2 is prominent but very transient, peaking already at 15 min of heat shock treatment at 42°C. This timing is concomitant with the acute response to heat stress, when the transcription of *Hsp* genes is escalating (1, 86). In contrast, HSF1 DNA-binding activity persisted throughout the time course studied. Interestingly, the decline in promoter occupancy of HSF2 coincided with the stress-induced ubiquitination kinetics. Therefore, it seems plausible that the destruction of HSF2 should have direct effects on the occupation of HSF2 on the heat shock-activated promoters. However, to accomplish such an immediate and strong effect on the DNA-binding portion of a transcription factor, the proteins regulating ubiquitination should be spatiotemporally available for the target (65). In the case of *Hsp70*, Cdc20, which may promote HSF2 ubiquitin conjugation, was detected at the promoter during heat shock. Turnell and coworkers have demonstrated a CBP/p300-dependent recruitment of APC/C subunits to specific promoters, where APC/C together with the acetyltransferase CBP/p300 regulates transcription (92). To our knowledge, our study is the first to report localization of Cdc20 on a transcriptionally active promoter, where it could target a transcription factor for degradation. Interestingly, the proteasome subunit $\alpha 2$ was also found at the promoter during heat shock, indicating that the DNA-bound HSF2 is directly targeted for degradation by APC/C and that the amount of HSF2 present at the promoter during the first 30 min of moderate heat shock is critical in mammalian cells. Remarkably, when Cdc20 was silenced, the regulation of HSF2 promoter occupancy on the *Hsp70* promoter was abolished, thereby altering HSF2 DNA-binding kinetics.

Both HSF1 and HSF2 bind DNA as trimers, and as recently demonstrated, these transcription factors also bind DNA in a heteromeric complex (58, 82). The concentration of HSF2 is regulated specifically under various circumstances, such as upon stress or during development (14, 59, 78, 82). Indeed, the expression of HSF2 is regulated by microRNA miR-18 in a cell-type-specific manner during spermatogenesis, resulting in altered expression of HSF2 target genes (13). Presumably, at certain stages of development when HSF2 is abundantly expressed, more HSF1-HSF2 heterotrimers than homotrimers are formed, whereas in stressed cells the levels of HSF2 are downregulated, resulting in fewer heterotrimers (for a model, see reference 82). Because HSF2 binding to the *Hsp* promoters is strictly HSF1 dependent in response to stress, the heat shock-induced degradation of HSF2 may change the composition of trimers occupying the *Hsp70* promoter. This regulatory step of HSF2 concentration could have important consequences for the stress response, which is evident in cells lacking HSF2, which exhibit perturbed *Hsp* transcription (68, 82). In

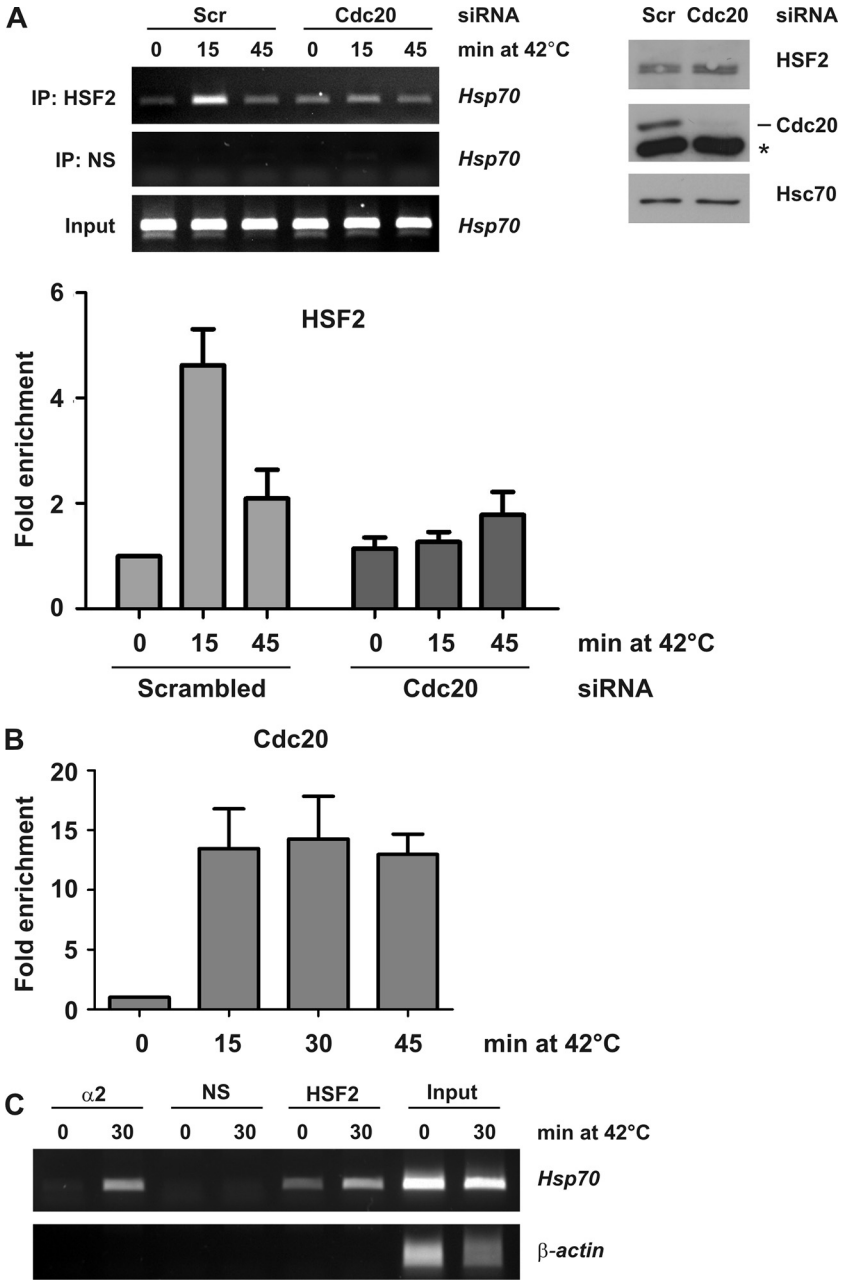

FIG. 6. Cdc20 and proteasome subunit α2 are recruited to the *Hsp70* promoter in response to heat stress. (A) ChIP for HSF2 binding to the *Hsp70* promoter was performed on HeLa cells transfected with scrambled (Scr) or Cdc20 siRNA, followed by heat shock treatment at 42°C for the indicated times. The top left panel shows a representative ChIP experiment, where NS denotes a nonspecific antibody. The samples were analyzed as described in the legend for Fig. 1A except that the value of binding at the 0-min time point in the scrambled siRNA-transfected cells was set to 1 (bottom left). Error bars represent standard errors of the means from four independent experiments. For the representative blot of protein levels shown in the right panel, Laemmli samples of the transfected cells were collected prior to heat shock treatment and run on SDS-polyacrylamide gels, followed by immunoblotting with antibodies for HSF2, Cdc20, and Hsc70. *, unspecific band. (B) K562 cells were heat shocked at 42°C for the indicated times, and four independent experiments were analyzed by ChIP with anti-Cdc20 antibody and quantitative real-time PCR as described above. (C) ChIP with antibody against the α2 subunit of the proteasome was performed on K562 cells that were left untreated or treated with heat shock for 30 min at 42°C. NS denotes a nonspecific antibody control. The IP and input samples were subjected to PCR using primers specific for the human *Hsp70* and β-*actin* promoters.

the present study, the dynamics of HSF2 binding to the *Hsp70* promoter was markedly altered when Cdc20 was silenced. Consequently, Cdc20 depletion led to increased *Hsp70* transcription in cells exposed to heat shock, which is in agreement with the response obtained in HSF2$^{-/-}$ cells, where the lack of HSF2 impairs heat shock-induced transcription of *Hsp70* (68). Underlining the relevance of Cdc20 in the heat shock response, the transcription of *Sat III* repeats was decreased in Cdc20-silenced cells. This result is in line with previous data showing that the heat shock-induced transcriptional activity in

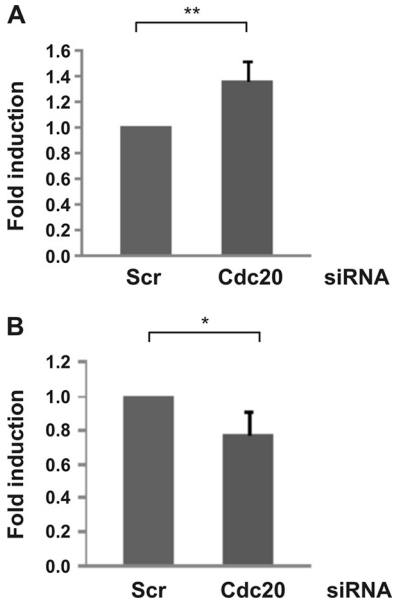

FIG. 7. Altered transcription of *Hsp70* and *Sat III* upon Cdc20 knockdown. HeLa cells were transfected with scrambled (Scr) and Cdc20 siRNA oligonucleotides and heat shocked for 1 h at 42°C. The mRNAs for *Hsp70* (A) and *Satellite III* (B) were determined by quantitative real-time RT-PCR. The relative expression levels were calculated, and the value for the scrambled control was set to 1. Error bars represent standard deviations from five (A) or four (B) independent experiments. *, $P < 0.05$; **, $P < 0.01$. A representative blot for Cdc20 knockdown is shown in Fig. 6A.

nSBs was increased upon siRNA-mediated HSF2 knockdown, highlighting the significance of HSF2 concentration for proper transcriptional regulation in a target-specific manner (82).

According to our working hypothesis of the activation of HSFs in response to moderate heat stress, APC/C^{Cdc20} is recruited to the *Hsp70* promoter, which is occupied by both HSF1 and HSF2. Yet, APC/C may facilitate the ubiquitination and proteasomal degradation specifically of HSF2, which would enable fine-tuning of the amount of HSF1 and HSF2 occupying the promoter. In the absence of Cdc20, the discrete HSF2 binding kinetics at the *Hsp70* promoter is disturbed, which might result in inappropriate transcription. The regulation of HSF2 concentration might provide a means to control the different phases of the stress-induced activation cycle so that the presence of HSF2 is important for the initiation phase, whereas an elimination of HSF2 from the target promoter makes HSF1 deactivation and attenuation possible. In summary, our study provides the first evidence for a new role of APC/C in regulating promoter dynamics in the acute response to protein-damaging stress.

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REFERENCES

1. **Abravaya, K., B. Phillips, and R. I. Morimoto.** 1991. Attenuation of the heat shock response in HeLa cells is mediated by the release of bound heat shock transcription factor and is modulated by changes in growth and in heat shock temperatures. Genes Dev. **5:**2117–2127.
2. **Adhikary, S., F. Marinoni, A. Hock, E. Hulleman, N. Popov, R. Beier, S. Bernard, M. Quarto, M. Capra, S. Goettig, U. Kogel, M. Scheffner, K. Helin, and M. Eilers.** 2005. The ubiquitin ligase HectH9 regulates transcriptional activation by Myc and is essential for tumor cell proliferation. Cell **123:**409–421.
3. **Ahn, S. G., P. C. Liu, K. Klyachko, R. I. Morimoto, and D. J. Thiele.** 2001. The loop domain of heat shock transcription factor 1 dictates DNA-binding specificity and responses to heat stress. Genes Dev. **15:**2134–2145.
4. **Ahn, S. G., and D. J. Thiele.** 2003. Redox regulation of mammalian heat shock factor 1 is essential for Hsp gene activation and protection from stress. Genes Dev. **17:**516–528.
5. **Åkerfelt, M., E. Henriksson, A. Laiho, A. Vihervaara, K. Rautoma, N. Kotaja, and L. Sistonen.** 2008. Promoter ChIP-chip analysis in mouse testis reveals Y chromosome occupancy by HSF2. Proc. Natl. Acad. Sci. U. S. A. **105:**11224–11229.
6. **Åkerfelt, M., R. I. Morimoto, and L. Sistonen.** 2010. Heat shock factors: integrators of cell stress, development and lifespan. Nat. Rev. Mol. Cell Biol. **11:**545–555.
7. **Alastalo, T. P., M. Hellesuo, A. Sandqvist, V. Hietakangas, M. Kallio, and L. Sistonen.** 2003. Formation of nuclear stress granules involves HSF2 and coincides with the nucleolar localization of Hsp70. J. Cell Sci. **116:**3557–3570.
8. **Almeida, A., J. P. Bolanos, and S. Moreno.** 2005. Cdh1/Hct1-APC is essential for the survival of postmitotic neurons. J. Neurosci. **25:**8115–8121.
9. **Anckar, J., and L. Sistonen.** 2007. Heat shock factor 1 as a coordinator of stress and developmental pathways. Adv. Exp. Med. Biol. **594:**78–88.
10. **Bennett, E. J., T. A. Shaler, B. Woodman, K. Y. Ryu, T. S. Zaitseva, C. H. Becker, G. P. Bates, H. Schulman, and R. R. Kopito.** 2007. Global changes to the ubiquitin system in Huntington's disease. Nature **448:**704–708.
11. **Biamonti, G.** 2004. Nuclear stress bodies: a heterochromatin affair? Nat. Rev. Mol. Cell Biol. **5:**493–498.
12. **Biggs, J. R., L. F. Peterson, Y. Zhang, A. S. Kraft, and D. E. Zhang.** 2006. AML1/RUNX1 phosphorylation by cyclin-dependent kinases regulates the degradation of AML1/RUNX1 by the anaphase-promoting complex. Mol. Cell. Biol. **26:**7420–7429.
13. **Björk, J. K., A. Sandqvist, A. N. Elsing, N. Kotaja, and L. Sistonen.** 2010. miR-18, a member of Oncomir-1, targets heat shock transcription factor 2 in spermatogenesis. Development **137:**3177–3184.
14. **Chang, Y., P. Östling, M. Åkerfelt, D. Trouillet, M. Rallu, Y. Gitton, R. El Fatimy, V. Fardeau, S. Le Crom, M. Morange, L. Sistonen, and V. Mezger.** 2006. Role of heat-shock factor 2 in cerebral cortex formation and as a regulator of p35 expression. Genes Dev. **20:**836–847.
15. **Chastagner, P., A. Israel, and C. Brou.** 2006. Itch/AIP4 mediates Deltex degradation through the formation of K29-linked polyubiquitin chains. EMBO Rep. **7:**1147–1153.
16. **Ciechanover, A.** 2005. Proteolysis: from the lysosome to ubiquitin and the proteasome. Nat. Rev. Mol. Cell Biol. **6:**79–87.
17. **Ciechanover, A., D. Finley, and A. Varshavsky.** 1984. Ubiquitin dependence of selective protein degradation demonstrated in the mammalian cell cycle mutant ts85. Cell **37:**57–66.
18. **Clute, P., and J. Pines.** 1999. Temporal and spatial control of cyclin B1 destruction in metaphase. Nat. Cell Biol. **1:**82–87.
19. **Corey, L. L., C. S. Weirich, I. J. Benjamin, and R. E. Kingston.** 2003. Localized recruitment of a chromatin-remodeling activity by an activator in vivo drives transcriptional elongation. Genes Dev. **17:**1392–1401.
20. **Cripps, D., S. N. Thomas, Y. Jeng, F. Yang, P. Davies, and A. J. Yang.** 2006. Alzheimer disease-specific conformation of hyperphosphorylated paired helical filament-Tau is polyubiquitinated through Lys-48, Lys-11, and Lys-6 ubiquitin conjugation. J. Biol. Chem. **281:**10825–10838.
21. **Deng, L., C. Wang, E. Spencer, L. Yang, A. Braun, J. You, C. Slaughter, C. Pickart, and Z. J. Chen.** 2000. Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. Cell **103:**351–361.
22. **Fang, G., H. Yu, and M. W. Kirschner.** 1998. The checkpoint protein MAD2 and the mitotic regulator CDC20 form a ternary complex with the anaphase-promoting complex to control anaphase initiation. Genes Dev. **12:**1871–1883.
23. **Fang, G., H. Yu, and M. W. Kirschner.** 1998. Direct binding of CDC20 protein family members activates the anaphase-promoting complex in mitosis and G1. Mol. Cell **2:**163–171.
24. **Finley, D., A. Ciechanover, and A. Varshavsky.** 1984. Thermolability of ubiquitin-activating enzyme from the mammalian cell cycle mutant ts85. Cell **37:**43–55.
25. **Fiorenza, M. T., T. Farkas, M. Dissing, D. Kolding, and V. Zimarino.** 1995.

Complex expression of murine heat shock transcription factors. Nucleic Acids Res. **23:**467–474.
26. **Floyd, S., J. Pines, and C. Lindon.** 2008. APC/C Cdh1 targets aurora kinase to control reorganization of the mitotic spindle at anaphase. Curr. Biol. **18:**1649–1658.
27. **Fraschini, R., A. Beretta, L. Sironi, A. Musacchio, G. Lucchini, and S. Piatti.** 2001. Bub3 interaction with Mad2, Mad3 and Cdc20 is mediated by WD40 repeats and does not require intact kinetochores. EMBO J. **20:**6648–6659.
28. **Gabellini, D., I. N. Colaluca, H. C. Vodermaier, G. Biamonti, M. Giacca, A. Falaschi, S. Riva, and F. A. Peverali.** 2003. Early mitotic degradation of the homeoprotein HOXC10 is potentially linked to cell cycle progression. EMBO J. **22:**3715–3724.
29. **Garcia-Higuera, I., E. Manchado, P. Dubus, M. Canamero, J. Mendez, S. Moreno, and M. Malumbres.** 2008. Genomic stability and tumour suppression by the APC/C cofactor Cdh1. Nat. Cell Biol. **10:**802–811.
30. **Geetha, T., J. Jiang, and M. W. Wooten.** 2005. Lysine 63 polyubiquitination of the nerve growth factor receptor TrkA directs internalization and signaling. Mol. Cell **20:**301–312.
31. **Geley, S., E. Kramer, C. Gieffers, J. Gannon, J. M. Peters, and T. Hunt.** 2001. Anaphase-promoting complex/cyclosome-dependent proteolysis of human cyclin A starts at the beginning of mitosis and is not subject to the spindle assembly checkpoint. J. Cell Biol. **153:**137–148.
32. **Guettouche, T., F. Boellmann, W. S. Lane, and R. Voellmy.** 2005. Analysis of phosphorylation of human heat shock factor 1 in cells experiencing a stress. BMC Biochem. **6:**4.
33. **Gurden, M. D., A. J. Holland, W. van Zon, A. Tighe, M. A. Vergnolle, D. A. Andres, H. P. Spielmann, M. Malumbres, R. M. Wolthuis, D. W. Cleveland, and S. S. Taylor.** 2010. Cdc20 is required for the post-anaphase, KEN-dependent degradation of centromere protein F. J. Cell Sci. **123:**321–330.
34. **Hagting, A., N. Den Elzen, H. C. Vodermaier, I. C. Waizenegger, J. M. Peters, and J. Pines.** 2002. Human securin proteolysis is controlled by the spindle checkpoint and reveals when the APC/C switches from activation by Cdc20 to Cdh1. J. Cell Biol. **157:**1125–1137.
35. **Haupt, Y., R. Maya, A. Kazaz, and M. Oren.** 1997. Mdm2 promotes the rapid degradation of p53. Nature **387:**296–299.
36. **He, H., F. Soncin, N. Grammatikakis, Y. Li, A. Siganou, J. Gong, S. A. Brown, R. E. Kingston, and S. K. Calderwood.** 2003. Elevated expression of heat shock factor (HSF) 2A stimulates HSF1-induced transcription during stress. J. Biol. Chem. **278:**35465–35475.
37. **Hietakangas, V., J. K. Ahlskog, A. M. Jakobsson, M. Hellesuo, N. M. Sahlberg, C. I. Holmberg, A. Mikhailov, J. J. Palvimo, L. Pirkkala, and L. Sistonen.** 2003. Phosphorylation of serine 303 is a prerequisite for the stress-inducible SUMO modification of heat shock factor 1. Mol. Cell. Biol. **23:**2953–2968.
38. **Holmberg, C. I., V. Hietakangas, A. Mikhailov, J. O. Rantanen, M. Kallio, A. Meinander, J. Hellman, N. Morrice, C. MacKintosh, R. I. Morimoto, J. E. Eriksson, and L. Sistonen.** 2001. Phosphorylation of serine 230 promotes inducible transcriptional activity of heat shock factor 1. EMBO J. **20:**3800–3810.
39. **Holmberg, C. I., S. A. Illman, M. Kallio, A. Mikhailov, and L. Sistonen.** 2000. Formation of nuclear HSF1 granules varies depending on stress stimuli. Cell Stress Chaperones **5:**219–228.
40. **Hong, Y., R. Rogers, M. J. Matunis, C. N. Mayhew, M. L. Goodson, O. K. Park-Sarge, and K. D. Sarge.** 2001. Regulation of heat shock transcription factor 1 by stress-induced SUMO-1 modification. J. Biol. Chem. **276:**40263–40267.
41. **Inouye, S., K. Katsuki, H. Izu, M. Fujimoto, K. Sugahara, S. Yamada, Y. Shinkai, Y. Oka, Y. Katoh, and A. Nakai.** 2003. Activation of heat shock genes is not necessary for protection by heat shock transcription factor 1 against cell death due to a single exposure to high temperatures. Mol. Cell. Biol. **23:**5882–5895.
42. **Jin, L., A. Williamson, S. Banerjee, I. Philipp, and M. Rape.** 2008. Mechanism of ubiquitin-chain formation by the human anaphase-promoting complex. Cell **133:**653–665.
43. **Jolly, C., L. Konecny, D. L. Grady, Y. A. Kutskova, J. J. Cotto, R. I. Morimoto, and C. Vourc'h.** 2002. In vivo binding of active heat shock transcription factor 1 to human chromosome 9 heterochromatin during stress. J. Cell Biol. **156:**775–781.
44. **Kallio, M., J. Weinstein, J. R. Daum, D. J. Burke, and G. J. Gorbsky.** 1998. Mammalian p55CDC mediates association of the spindle checkpoint protein Mad2 with the cyclosome/anaphase-promoting complex, and is involved in regulating anaphase onset and late mitotic events. J. Cell Biol. **141:**1393–1406.
45. **Kawazoe, Y., A. Nakai, M. Tanabe, and K. Nagata.** 1998. Proteasome inhibition leads to the activation of all members of the heat-shock-factor family. Eur. J. Biochem. **255:**356–362.
46. **Kim, A. H., S. V. Puram, P. M. Bilimoria, Y. Ikeuchi, S. Keough, M. Wong, D. Rowitch, and A. Bonni.** 2009. A centrosomal Cdc20-APC pathway controls dendrite morphogenesis in postmitotic neurons. Cell **136:**322–336.
47. **Kim, T. K., and T. Maniatis.** 1996. Regulation of interferon-gamma-activated STAT1 by the ubiquitin-proteasome pathway. Science **273:**1717–1719.
48. **King, R. W., J. M. Peters, S. Tugendreich, M. Rolfe, P. Hieter, and M. W. Kirschner.** 1995. A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. Cell **81:**279–288.
49. **Kline, M. P., and R. I. Morimoto.** 1997. Repression of the heat shock factor 1 transcriptional activation domain is modulated by constitutive phosphorylation. Mol. Cell. Biol. **17:**2107–2115.
50. **Konishi, Y., J. Stegmuller, T. Matsuda, S. Bonni, and A. Bonni.** 2004. Cdh1-APC controls axonal growth and patterning in the mammalian brain. Science **303:**1026–1030.
51. **Kraft, C., M. Gmachl, and J. M. Peters.** 2006. Methods to measure ubiquitin-dependent proteolysis mediated by the anaphase-promoting complex. Methods **38:**39–51.
52. **Kramer, E. R., C. Gieffers, G. Holzl, M. Hengstschlager, and J. M. Peters.** 1998. Activation of the human anaphase-promoting complex by proteins of the CDC20/Fizzy family. Curr. Biol. **8:**1207–1210.
53. **Lasorella, A., J. Stegmuller, D. Guardavaccaro, G. Liu, M. S. Carro, G. Rothschild, L. de la Torre-Ubieta, M. Pagano, A. Bonni, and A. Iavarone.** 2006. Degradation of Id2 by the anaphase-promoting complex couples cell cycle exit and axonal growth. Nature **442:**471–474.
54. **Lee, Y. J., H. J. Lee, J. S. Lee, D. Jeoung, C. M. Kang, S. Bae, S. J. Lee, S. H. Kwon, D. Kang, and Y. S. Lee.** 2008. A novel function for HSF1-induced mitotic exit failure and genomic instability through direct interaction between HSF1 and Cdc20. Oncogene **27:**2999–3009.
55. **Li, M., Y. H. Shin, L. Hou, X. Huang, Z. Wei, E. Klann, and P. Zhang.** 2008. The adaptor protein of the anaphase promoting complex Cdh1 is essential in maintaining replicative lifespan and in learning and memory. Nat. Cell Biol. **10:**1083–1089.
56. **Lindon, C., and J. Pines.** 2004. Ordered proteolysis in anaphase inactivates Plk1 to contribute to proper mitotic exit in human cells. J. Cell Biol. **164:**233–241.
57. **Lo, R. S., and J. Massague.** 1999. Ubiquitin-dependent degradation of TGF-beta-activated smad2. Nat. Cell Biol. **1:**472–478.
58. **Loison, F., L. Debure, P. Nizard, P. le Goff, D. Michel, and Y. le Drean.** 2006. Up-regulation of the clusterin gene after proteotoxic stress: implication of HSF1-HSF2 heterocomplexes. Biochem. J. **395:**223–231.
59. **Mathew, A., S. K. Mathur, and R. I. Morimoto.** 1998. Heat shock response and protein degradation: regulation of HSF2 by the ubiquitin-proteasome pathway. Mol. Cell. Biol. **18:**5091–5098.
60. **Maxwell, P. H., M. S. Wiesener, G. W. Chang, S. C. Clifford, E. C. Vaux, M. E. Cockman, C. C. Wykoff, C. W. Pugh, E. R. Maher, and P. J. Ratcliffe.** 1999. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature **399:**271–275.
61. **McMillan, D. R., X. Xiao, L. Shao, K. Graves, and I. J. Benjamin.** 1998. Targeted disruption of heat shock transcription factor 1 abolishes thermotolerance and protection against heat-inducible apoptosis. J. Biol. Chem. **273:**7523–7528.
62. **Morimoto, R. I.** 2008. Proteotoxic stress and inducible chaperone networks in neurodegenerative disease and aging. Genes Dev. **22:**1427–1438.
63. **Morris, J. R., and E. Solomon.** 2004. BRCA1 : BARD1 induces the formation of conjugated ubiquitin structures, dependent on K6 of ubiquitin, in cells during DNA replication and repair. Hum. Mol. Genet. **13:**807–817.
64. **Murata, S., H. Yashiroda, and K. Tanaka.** 2009. Molecular mechanisms of proteasome assembly. Nat. Rev. Mol. Cell Biol. **10:**104–115.
65. **Muratani, M., and W. P. Tansey.** 2003. How the ubiquitin-proteasome system controls transcription. Nat. Rev. Mol. Cell Biol. **4:**192–201.
66. **Murray, A. W.** 2004. Recycling the cell cycle: cyclins revisited. Cell **116:**221–234.
67. **Nishikawa, H., S. Ooka, K. Sato, K. Arima, J. Okamoto, R. E. Klevit, M. Fukuda, and T. Ohta.** 2004. Mass spectrometric and mutational analyses reveal Lys-6-linked polyubiquitin chains catalyzed by BRCA1-BARD1 ubiquitin ligase. J. Biol. Chem. **279:**3916–3924.
68. **Östling, P., J. K. Björk, P. Roos-Mattjus, V. Mezger, and L. Sistonen.** 2007. Heat shock factor 2 (HSF2) contributes to inducible expression of hsp genes through interplay with HSF1. J. Biol. Chem. **282:**7077–7086.
69. **Park, H. J., R. H. Costa, L. F. Lau, A. L. Tyner, and P. Raychaudhuri.** 2008. Anaphase-promoting complex/cyclosome-CDH1-mediated proteolysis of the forkhead box M1 transcription factor is critical for regulated entry into S phase. Mol. Cell. Biol. **28:**5162–5171.
70. **Peters, J. M.** 2006. The anaphase promoting complex/cyclosome: a machine designed to destroy. Nat. Rev. Mol. Cell Biol. **7:**644–656.
71. **Pfleger, C. M., and M. W. Kirschner.** 2000. The KEN box: an APC recognition signal distinct from the D box targeted by Cdh1. Genes Dev. **14:**655–665.
72. **Pfleger, C. M., E. Lee, and M. W. Kirschner.** 2001. Substrate recognition by the Cdc20 and Cdh1 components of the anaphase-promoting complex. Genes Dev. **15:**2396–2407.
73. **Pickart, C. M.** 2004. Back to the future with ubiquitin. Cell **116:**181–190.
74. **Pickart, C. M., and R. E. Cohen.** 2004. Proteasomes and their kin: proteases in the machine age. Nat. Rev. Mol. Cell Biol. **5:**177–187.

75. **Pickart, C. M., and D. Fushman.** 2004. Polyubiquitin chains: polymeric protein signals. Curr. Opin. Chem. Biol. **8:**610–616.
76. **Pirkkala, L., T. P. Alastalo, X. Zuo, I. J. Benjamin, and L. Sistonen.** 2000. Disruption of heat shock factor 1 reveals an essential role in the ubiquitin proteolytic pathway. Mol. Cell. Biol. **20:**2670–2675.
77. **Prinz, S., E. S. Hwang, R. Visintin, and A. Amon.** 1998. The regulation of Cdc20 proteolysis reveals a role for APC components Cdc23 and Cdc27 during S phase and early mitosis. Curr. Biol. **8:**750–760.
78. **Rallu, M., M. Loones, Y. Lallemand, R. Morimoto, M. Morange, and V. Mezger.** 1997. Function and regulation of heat shock factor 2 during mouse embryogenesis. Proc. Natl. Acad. Sci. U. S. A. **94:**2392–2397.
79. **Ravid, T., and M. Hochstrasser.** 2008. Diversity of degradation signals in the ubiquitin-proteasome system. Nat. Rev. Mol. Cell Biol. **9:**679–690.
80. **Rodrigo-Brenni, M. C., and D. O. Morgan.** 2007. Sequential E2s drive polyubiquitin chain assembly on APC targets. Cell **130:**127–139.
81. **Salghetti, S. E., M. Muratani, H. Wijnen, B. Futcher, and W. P. Tansey.** 2000. Functional overlap of sequences that activate transcription and signal ubiquitin-mediated proteolysis. Proc. Natl. Acad. Sci. U. S. A. **97:**3118–3123.
82. **Sandqvist, A., J. K. Björk, M. Åkerfelt, Z. Chitikova, A. Grichine, C. Vourc'h, C. Jolly, T. A. Salminen, Y. Nymalm, and L. Sistonen.** 2009. Heterotrimerization of HSF1 and HSF2 provides a transcriptional switch in response to distinct stimuli. Mol. Biol. Cell **20:**1340–1347.
83. **Sarge, K. D., S. P. Murphy, and R. I. Morimoto.** 1993. Activation of heat shock gene transcription by heat shock factor 1 involves oligomerization, acquisition of DNA-binding activity, and nuclear localization and can occur in the absence of stress. Mol. Cell. Biol. **13:**1392–1407.
84. **Sheldon, L. A., and R. E. Kingston.** 1993. Hydrophobic coiled-coil domains regulate the subcellular localization of human heat shock factor 2. Genes Dev. **7:**1549–1558.
85. **Shi, Y., D. D. Mosser, and R. I. Morimoto.** 1998. Molecular chaperones as HSF1-specific transcriptional repressors. Genes Dev. **12:**654–666.
86. **Sistonen, L., K. D. Sarge, and R. I. Morimoto.** 1994. Human heat shock factors 1 and 2 are differentially activated and can synergistically induce hsp70 gene transcription. Mol. Cell. Biol. **14:**2087–2099.
87. **Sudakin, V., G. K. Chan, and T. J. Yen.** 2001. Checkpoint inhibition of the APC/C in HeLa cells is mediated by a complex of BUBR1, BUB3, CDC20, and MAD2. J. Cell Biol. **154:**925–936.
88. **Sudakin, V., D. Ganoth, A. Dahan, H. Heller, J. Hershko, F. C. Luca, J. V. Ruderman, and A. Hershko.** 1995. The cyclosome, a large complex containing cyclin-selective ubiquitin ligase activity, targets cyclins for destruction at the end of mitosis. Mol. Biol. Cell **6:**185–197.
89. **Tang, Z., R. Bharadwaj, B. Li, and H. Yu.** 2001. Mad2-independent inhibition of APCCdc20 by the mitotic checkpoint protein BubR1. Dev. Cell **1:**227–237.
90. **Tang, Z., H. Shu, D. Oncel, S. Chen, and H. Yu.** 2004. Phosphorylation of Cdc20 by Bub1 provides a catalytic mechanism for APC/C inhibition by the spindle checkpoint. Mol. Cell **16:**387–397.
91. **Thornton, B. R., and D. P. Toczyski.** 2006. Precise destruction: an emerging picture of the APC. Genes Dev. **20:**3069–3078.
92. **Turnell, A. S., G. S. Stewart, R. J. Grand, S. M. Rookes, A. Martin, H. Yamano, S. J. Elledge, and P. H. Gallimore.** 2005. The APC/C and CBP/p300 cooperate to regulate transcription and cell-cycle progression. Nature **438:**690–695.
93. **Valgardsdottir, R., I. Chiodi, M. Giordano, A. Rossi, S. Bazzini, C. Ghigna, S. Riva, and G. Biamonti.** 2008. Transcription of Satellite III non-coding RNAs is a general stress response in human cells. Nucleic Acids Res. **36:**423–434.
94. **van Roessel, P., D. A. Elliott, I. M. Robinson, A. Prokop, and A. H. Brand.** 2004. Independent regulation of synaptic size and activity by the anaphase-promoting complex. Cell **119:**707–718.
95. **Wang, C., L. Deng, M. Hong, G. R. Akkaraju, J. Inoue, and Z. J. Chen.** 2001. TAK1 is a ubiquitin-dependent kinase of MKK and IKK. Nature **412:**346–351.
96. **Weinstein, J.** 1997. Cell cycle-regulated expression, phosphorylation, and degradation of p55Cdc. A mammalian homolog of CDC20/Fizzy/slp1. J. Biol. Chem. **272:**28501–28511.
97. **Westerheide, S. D., J. Anckar, S. M. Stevens, Jr., L. Sistonen, and R. I. Morimoto.** 2009. Stress-inducible regulation of heat shock factor 1 by the deacetylase SIRT1. Science **323:**1063–1066.
98. **Wu, T., Y. Merbl, Y. Huo, J. L. Gallop, A. Tzur, and M. W. Kirschner.** 2010. UBE2S drives elongation of K11-linked ubiquitin chains by the anaphase-promoting complex. Proc. Natl. Acad. Sci. U. S. A. **107:**1355–1360.
99. **Xing, H., D. C. Wilkerson, C. N. Mayhew, E. J. Lubert, H. S. Skaggs, M. L. Goodson, Y. Hong, O. K. Park-Sarge, and K. D. Sarge.** 2005. Mechanism of hsp70i gene bookmarking. Science **307:**421–423.
100. **Yang, Y., A. H. Kim, T. Yamada, B. Wu, P. M. Bilimoria, Y. Ikeuchi, N. de la Iglesia, J. Shen, and A. Bonni.** 2009. A Cdc20-APC ubiquitin signaling pathway regulates presynaptic differentiation. Science **326:**575–578.
101. **Ye, Y., and M. Rape.** 2009. Building ubiquitin chains: E2 enzymes at work. Nat. Rev. Mol. Cell Biol. **10:**755–764.
102. **Yu, H.** 2007. Cdc20: a WD40 activator for a cell cycle degradation machine. Mol. Cell **27:**3–16.
103. **Yu, H., J. M. Peters, R. W. King, A. M. Page, P. Hieter, and M. W. Kirschner.** 1998. Identification of a cullin homology region in a subunit of the anaphase-promoting complex. Science **279:**1219–1222.
104. **Zachariae, W., A. Shevchenko, P. D. Andrews, R. Ciosk, M. Galova, M. J. Stark, M. Mann, and K. Nasmyth.** 1998. Mass spectrometric analysis of the anaphase-promoting complex from yeast: identification of a subunit related to cullins. Science **279:**1216–1219.
105. **Zur, A., and M. Brandeis.** 2001. Securin degradation is mediated by fzy and fzr, and is required for complete chromatid separation but not for cytokinesis. EMBO J. **20:**792–801.