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## **HERC2 interacts with Claspin and regulates DNA origin firing and replication fork progression**

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Running title: HERC2 regulates replication

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## Abstract

DNA replication, recombination and repair are highly interconnected processes the disruption of which must be coordinated in cancer. HERC2, a large HECT protein required for homologous recombination repair, is an E3 ubiquitin ligase that targets breast cancer suppressor BRCA1 for degradation. Here we show that HERC2 is a component of the DNA replication fork complex that plays a critical role in DNA elongation and origin firing. In the presence of BRCA1, endogenous HERC2 interacts with Claspin, a protein essential for G2/M checkpoint activation and replication fork stability. Claspin depletion slowed S-phase progression and additional HERC2 depletion reduced the effect of Claspin depletion. In addition, HERC2 interacts with replication fork complex proteins. Depletion of HERC2 alleviated the slow replication fork progression in Claspin-deficient cells, suppressed enhanced origin firing, and led to a decrease in MCM2 phosphorylation. In a HERC2-dependent manner, treatment of cells with replication inhibitor aphidicolin enhanced MCM2 phosphorylation. Taken together, our results suggest that HERC2 regulates DNA replication progression and origin firing by facilitating MCM2 phosphorylation. These findings establish HERC2 as a critical function in DNA repair, checkpoint activation and DNA replication.

## Introduction

DNA replication, recombination and repair coordinately maintain genome stability, and their defect is a hallmark of cancer cells. The DNA replication and damage response share many critical proteins. Among them are ATR-Chk1 pathway that is activated in response to stalled replication forks and prevents inappropriate entry into mitosis, while it also regulates normal DNA replication by stabilizing replication fork and inhibiting excess origin firing(1, 2). Claspin is a checkpoint mediator that facilitates the phosphorylation and activation of Chk1 by ATR(3). In addition Claspin in combination with TIPIN-TIM1-AND1 complex physically links the DNA polymerase and helicase activities, preventing fork collapse, and is required for a normal rate of fork progression(2, 4, 5).

HERC2 is a large HECT and RCC-like domain-containing protein comprising 4834 amino acids, and has recently been implicated in homologous recombination repair of DNA double-strand breaks (DSBs)(6). HERC2 is recruited to sites of DSBs and facilitates assembly of the RNF8-Ubc13 complex, and is thereby essential for ubiquitin-dependent retention of repair factors(6). HERC2 is also implicated in nucleotide excision repair by ubiquitinating and degrading XPA(7, 8). In addition we showed that HERC2 is an E3 ubiquitin ligase that targets BARD1-uncoupled BRCA1 for degradation(9). Depletion of HERC2 does not produce G2/M checkpoint failure(9) in spite of the fact that HERC2 is essential for recruiting the repair factors including BRCA1 that mediate G2/M checkpoint activation, to sites of DNA damage(6). Because depletion of HERC2 compensated for BRCA1 instability in BARD1 deficient cells and restored G2/M checkpoint function, we propose that HERC2 inhibits G2/M checkpoint

function by destabilizing BRCA1. HERC2 interacts with BRCA1 and may inhibit its G2/M checkpoint function during normal S-phase or during recovery from the checkpoint. However, role of HERC2 in normal S-phase is unknown.

Because BRCA1 interacts with Claspin and acts as a second regulator of Chk1 activation(10), HERC2 may interact with Claspin and regulate DNA replication. Here we show that HERC2 is a component of replication fork complex and regulates the fork progression and origin firing in conjunction with Claspin.

## **Materials and Methods**

### **Cell culture and transfection**

HCT116, HeLa, HEK293T, U2OS and either BRCA1-negative or -positive UWB1.289 cells were purchased from American Type Culture Collection, and cultured in DMEM supplemented with 10% FCS and 1% antibiotic-antimycotic agent. Cell lines were not further tested or authenticated. siRNA oligonucleotides targeting HERC2 (#1: 5'-GGAAAGCACUGGAUUCGUU-3' and #2: 5'-GAAGGUGGCUGUUCACUCA-3', sense strand), Claspin (#1: 5'-GGACGUAAUUGAUGAAGUA-3' and #2: 5'-GGAAAUACCUGGAGGAUGA-3', sense strand), and non-targeting control (D-001206-14) were purchased (Dharmacon). Myc-tagged N-terminus (1-2329, NT) and C-terminus (2292-4834, CT) of HERC2 fragments were subcloned in pcDNA3 by fusing smaller fragments of HERC2 described previously(9). Transfection was carried out as previously described(11). Aphidicolin was purchased (Sigma-Aldrich).

### **Cell cycle analysis**

Seventy-two hours after siRNA transfection HCT116 cells were treated with 100 ng/ml of nocodazole for 12 hours to trap cells in mitosis and to analyze single cycle of S-phase. Bromodeoxyuridine (BrdU) was added for last one hour. Cells were then harvested and DNA was stained with anti-BrdU mAb (Santa Cruz) and Propidium Iodide, and analyzed by flow cytometry as described elsewhere(12).

### **Immunoprecipitation, immunoblotting and immunocytochemistry**

Rabbit polyclonal antibodies to HERC2 (Bethyl Laboratories), Claspin (Bethyl Laboratories), ATRIP (Bethyl), phospho-MCM2-S108 (Bethyl Laboratories), Goat polyclonal antibodies to MCM2 (Santa Cruz) and mouse monoclonal antibodies (mAb) to HERC2 (BD Bioscience), MCM7 (Santa Cruz), PCNA (Neomarkers), TopBP1 (BD Bioscience),  $\alpha$ - and  $\beta$ -tubulin (DMIA+BMIB, Neomarkers), Actin (Sigma), and Myc (9E10, BabCo) were purchased commercially. Immunoprecipitation and immunoblotting were performed as described(11) with 0.5% NP-40-based lysis buffer in the presence or absence of Benzonase nuclease (Novagen) or with RIPA buffer for whole cell lysates(11, 13). For indirect immunocytochemistry cells were fixed with cold methanol for 60 minutes and permeablized with cold acetone for 5 seconds. Cells were then stained as previously described(13) with modification that blocking buffer contained 0.1% Triton X-100.

### **DNA fiber experiments**

Forty-eight hours after siRNA transfection, cells were pulse labeled with 25  $\mu$  M BrdU for indicated time length, followed by 250  $\mu$  M iododeoxyuridine (IdU) for 20 min. DNA combing was carried out as described elsewhere(14, 15) with modifications. Briefly,

3000 labeled cells spread on a glass slide was overlaid with 10  $\mu$ l of buffer containing 0.5% sodium dodecyl sulfate, 200 mM Tris-HCl (pH 7.4) and 50 mM EDTA. After 10 min, the slide was tilted at 30 degrees and the resulting DNA spreads were air-dried, fixed in 3:1 methanol/acetic acid for 5 min. The slides were treated with 2.5 M HCl for 60 min, washed in PBS, and blocked in 2% bovine serum albumin in PBS for 30 min. The DNA fibers were then immunostained with rat anti-BrdU mAb and mouse anti-BrdU/IdU mAb (BD Biosciences) followed by AlexaFluor 488-conjugated chicken anti-rat IgG (Invitrogen) and AlexaFluor 555-conjugated goat anti-mouse IgG (Millipore). Fluorescent-labeled fibers were then examined with an LSM 510 confocal microscope (Carl Zeiss). The lengths of BrdU- (green) and IdU (red)-labeled patches were measured using the LSM software (Carl Zeiss).

## Results and Discussion

### **HERC2 interacts with Claspin and affects S-phase progression.**

To examine whether HERC2 cooperates with Claspin, we first analyzed the interaction between endogenous HERC2 and Claspin by coupled immunoprecipitation and Western blotting. HERC2 was readily detected in Claspin immunocomplexes precipitated from HCT116 or HeLa cell lysate (Fig. 1A). The interaction was diminished in BRCA1-defective UWB 1.289 cells (Fig. 1B), suggesting that BRCA1 facilitates the interaction. We next examined whether HERC2 has some role in the effect of Claspin on S-phase progression. HCT116 cells were transfected with siRNA for Claspin and/or HERC2, and the S-phase progression was analyzed with BrdU. The siRNA treatment successfully inhibited expression of Claspin and HERC2 (Fig. 1C, Supplementary Fig.

S1). Depletion of Claspin expression increased cells in S-phase, including that in early S-phase (Fig. 1D), indicating slowed S-phase progression as previously reported (4). Importantly, additional HERC2 depletion with two different siRNAs both reduced the effect of Claspin depletion and decreased cells in S-phase to the level of control cells. This suggests that HERC2 suppresses S-phase progression in the absence of Claspin. Single HERC2 knockdown also slightly decreased cells in S-phase when compared to the control cells.

### **HERC2 localizes at DNA replication fork.**

We previously showed that HERC2 and BRCA1 co-localize at S-phase nuclear foci(9). To analyze whether HERC2 localizes at DNA replication foci, exponentially proliferating HeLa cells were immunostained with anti-HERC2 antibody in combination with antibodies to proteins in the replication fork complex. Interphase cells exhibited nuclear HERC2 foci as we demonstrated previously(9). Importantly clear co-localization of HERC2 with nuclear PCNA foci was visualized (Fig. 2A). The colocalization was especially remarkable in cells with larger PCNA foci (light upper cell in top panels), an indication of cells in late S-phase(16). To further demonstrate the interaction between HERC2 and PCNA, HEK-293T cells were transfected with HERC2 fragments. As shown in Figure 2B, PCNA were detected in the Myc-HERC2-CT immunocomplexes. Reciprocally, Myc-HERC2-CT was detected in PCNA immunocomplexes. The detection of the interaction was dependent on Endonuclease treatment of cell lysates, suggesting that HERC2 physically interacts with PCNA complex on chromatin. HERC2 also



colocalized at nuclear foci with TopBP1 and MCM7 (Fig. 2A). Together the results indicated that HERC2 is a component of DNA replication complex.

### **HERC2 suppresses DNA replication progression in the absence of Claspin.**

S-phase progression is regulated by two main mechanisms: replication origin firing and elongation. Claspin and Chk1 stabilize replication fork and maintain elongation while they suppress excess origin firing(5, 17). Therefore the observed effect on S-phase progression in cells depleted of Claspin and/or HERC2 (Fig. 1) could be a result from the two contradictory factors. To analyze this mechanism more precisely, we employed DNA combing experiments. The nascent DNAs were labeled with BrdU followed by IdU and the DNA lengths were analyzed with immunofluorescent detection. The reliability of the procedure was first verified by proportionate increase of the DNA length and labeling time length (Supplementary Fig. S2). HCT116 cells were then transfected with Claspin- or HERC2-specific siRNAs. The inhibition of HERC2 and/or Claspin expression was verified by Western blot (Fig. 3A, Supplementary Fig. S1). The lengths of the nascent DNAs in each sample were determined (Fig. 3B-D). Consistent with the previous report(4, 18), inhibition of Claspin shortened the DNA approximately one third of that in control cells (41.4 vs 14.0 or 14.7 kbp). Importantly depletion of HERC2 can alleviate the slow replication fork progression in the Claspin-deficient cells (14.0 vs 19.1 or 17.9 kbp). Interestingly however, single HERC2 knockdown slightly shortened, rather than lengthened, the DNA lengths (41.4 vs 35.6 or 36.5 kbp). The observed effect of HERC2 likely did not rely on its E3 activity for protein degradation, because proteasome inhibitor

MG132, instead of HERC2 deletion, did not affect the nascent DNA length shortened by Claspin inhibition (Supplementary Fig. S3).

### **HERC2 enhances origin firing in the absence of Claspin and facilitates MCM2 phosphorylation in response to replication stress**

Inhibition of Claspin leads to uncoupling of MCM helicase complex and polymerase complexes resulting in enhanced single-strand DNA (SSD) during replication elongation (5). Therefore the observed complement effect of HERC2 depletion on the slow replication fork progression in the Claspin-deficient cells could be explained by direct role of HERC2 on the fork stabilization. However, HERC2 depletion in the Claspin-deficient cells did not show obvious complementary effect on the foci formation of a SSD-binding protein RPA70 (Supplementary Fig. S4), suggesting that HERC2 does not directly involve in the fork stabilization.

We next analyzed the effect of HERC2 on origin firing. Origin firing is regulated by two contradictory mechanisms. Claspin and Chk1 inhibits excess origin firing while promotes elongation(17, 19). Contrary ATR-mediated phosphorylation of MCM2 recruits Plk1, which up regulates origin firing as a compensatory mechanism for survival of replication blocks(5, 20). Supporting this mechanism Claspin-deficient cells demonstrated enhanced origin firing (Fig. 4A). Significantly additional depletion of HERC2 re-suppressed the origin firing to the level of that in control cells. In addition depletion of Claspin enhanced the MCM2 phosphorylation and additional inhibition of HERC2 dramatically suppressed the MCM2 phosphorylation in the Claspin-deficient cells (Fig. 4B). To test the effect of HERC2 on replication stress in physiological

condition, we treated the cells with aphidicolin and ATRIP was immunoprecipitated. In addition to MCM2, HERC2 was co-precipitated from endonuclease-treated cell lysates (Fig. 4C, Supplementary Fig. S5). Importantly the aphidicolin treatment increased the amount of HERC2 in the ATRIP immunocomplex (Fig. 4C). In addition HERC2 depletion suppressed the MCM2 phosphorylation in the aphidicolin-treated cells (Fig. 4D), in similar fashion to that in the Claspin-deficient cells (Fig. 4B).

In summary, our results demonstrate that HERC2, an E3 ligase critical for DNA damage repair pathways, also regulates DNA replication progression and origin firing by facilitating MCM2 phosphorylation (Supplementary Fig. S6). Because HERC2 targets BRCA1 for degradation, it is possible that HERC2, Claspin and BRCA1 cooperate on activation of Chk1 and Plk1. HERC2 in maintenance of DNA stability warrants further study into its potential roles in cancer development and therapy.

### **Competing interests**

No potential conflicts of interest were disclosed.

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## FIGURE LEGENDS

**Figure 1.** HERC2 interacts with Claspin and affects S-phase progression. (A and B) Lysates prepared from HeLa, HCT116 (A) and BRCA1-negative and positive UWB 1.289 (B) cells were immunoprecipitated (IP) and immunoblotted (IB) with indicated antibodies. Inputs (1.5%) were also loaded. (C) HCT116 cells transfected with indicated siRNA were immunoblotted with indicated antibodies. Tubulin: loading control. (D) Cells from (C) were analyzed for cell cycle. The percentages of early S- (indicated with square boxes) and S-phase cells are shown in bottom. CTR: control.

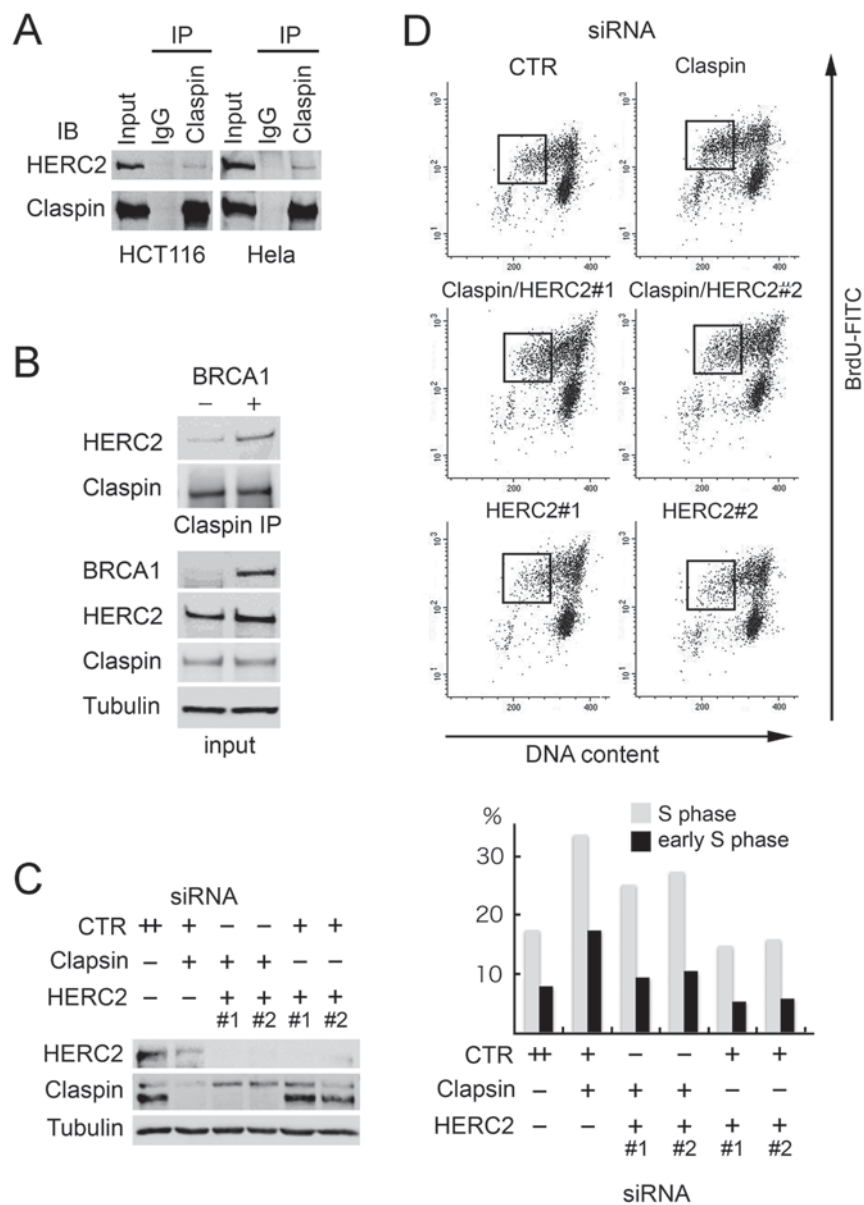
**Figure 2.** HERC2 localizes at DNA replication foci. (A) HeLa cells were immunostained with anti-HERC2 antibody in combination with indicated antibodies. The nucleus was counterstained with DAPI. (B) HEK-293T cells were transfected with Myc-HERC2-NT or CT fragment, or parental pcDNA3 vector, and lysed with or without endonuclease. Interaction between Myc-HERC2 fragments and PCNA were assessed by immunoprecipitation (IP) followed by immunoblotting (IB) with indicated antibodies.

**Figure 3.** HERC2 suppresses DNA strand elongation in the absence of Claspin. (A and B) HCT116 cells transfected with the indicated siRNA were subjected to immunoblot (A) or sequentially treated with BrdU and IdU for 20 min each to label the nascent DNAs (B). (C) Representative images of labeled DNA fibers from cells with indicated siRNA measured with confocal microscopy. (D) Distributions of replication fork length during the entire labeling period in cells with the indicated siRNA are shown with the mean percentages (bars). Significance was analyzed by Student's t-test. \* $p < .0001$

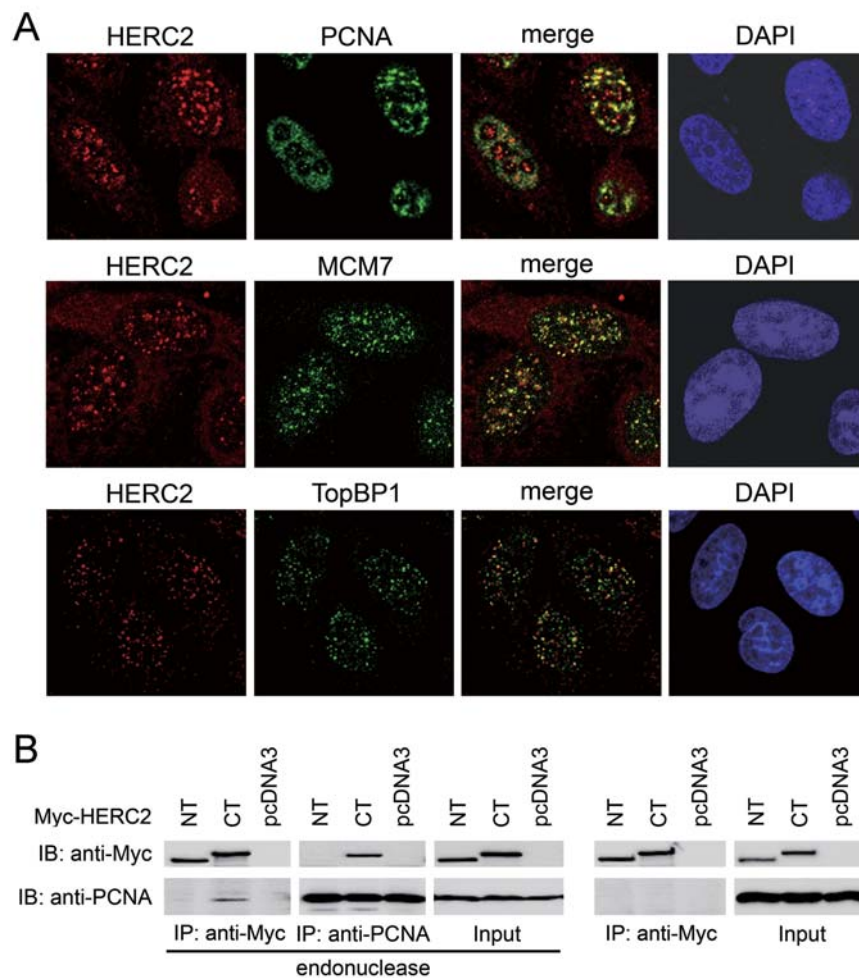
**Figure 4.** HERC2 enhances origin firing and MCM2 phosphorylation in replication stress. (A) The nascent DNAs were labeled as in Figure 3. Representative images of the labeled DNA fibers of each origin-firing or elongation pattern from control cells are shown. Arrows indicate the position of replication initiation. Bottom panel: Quantification of origin firing in cells with the indicated siRNA. Summation of number of first label origins (red-green-red), second label origins (red only), and interspersed patterns (repeated red-green-red) are shown as percentage of all labeled tracks. Data represents the mean of triplicate experimental samples and error bars indicate standard deviation. Significance was analyzed by Student's t-test. (B) HCT116 cells were transfected with the indicated siRNA and whole cell lysates were immunoblotted with indicated antibodies. Aphidicolin (Aph, 4  $\mu$ M) was added for three hours as a positive control for MCM2 phosphorylation. (C) HeLa cells untreated or treated with 4 $\mu$ M aphidicolin for 3 hours were immunoprecipitated (IP) in the presence of endonuclease, and immunoblotted (IB) with indicated antibodies. (D) HCT116 cells were transfected with the indicated siRNA, untreated or treated with 1 $\mu$ M aphidicolin for 3 hours, and whole cell lysates were immunoblotted with indicated antibodies. Asterisk: phosphorylated MCM2.



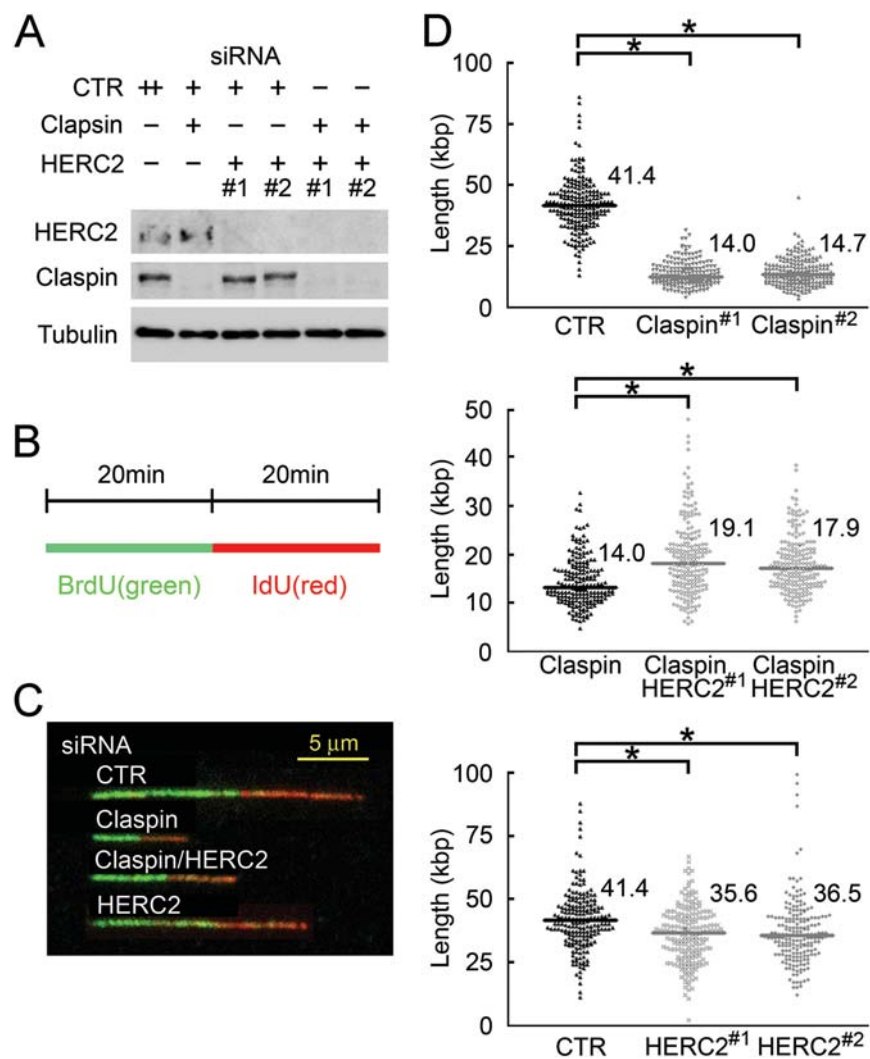
Izawa, et al. Figure 1



Izawa, et al. **Figure 2**



Izawa, et al. Figure 3



Izawa, et al. Figure 4

