Overexpression of Claspin and Timeless protects cancer cells from replication stress in a checkpoint-independent manner

Julien N. Bianco1,7, Valérie Bergoglio2, Yea-Lih Lin1, Marie-Jeanne Pillaire2, Anne-Lyne Schmitz1, Julia Gilhodes3, Amelie Lusque3, Julien Mazières4, Magali Lacroix-Triki5, Theodoros I. Roumeliotis6, Jyoti Choudhary6, Jérôme Moreaux1, Jean-Sébastien Hoffmann2, Hélène Tourrière1 & Philippe Pasero1

Oncogene-induced replication stress (RS) promotes cancer development but also impedes tumor growth by activating anti-cancer barriers. To determine how cancer cells adapt to RS, we have monitored the expression of different components of the ATR-CHK1 pathway in primary tumor samples. We show that unlike upstream components of the pathway, the checkpoint mediators Claspin and Timeless are overexpressed in a coordinated manner. Remarkably, reducing the levels of Claspin and Timeless in HCT116 cells to pretumoral levels impeded fork progression without affecting checkpoint signaling. These data indicate that high level of Claspin and Timeless increase RS tolerance by protecting replication forks in cancer cells. Moreover, we report that primary fibroblasts adapt to oncogene-induced RS by spontaneously overexpressing Claspin and Timeless, independently of ATR signaling. Altogether, these data indicate that enhanced levels of Claspin and Timeless represent a gain of function that protects cancer cells from of oncogene-induced RS in a checkpoint-independent manner.
Genomic instability is a cancer hallmark that is detected at early stages of tumorigenesis and is generally considered as a driving force of cancer development. A growing body of evidence indicates that DNA damage arises as a consequence of oncogene-induced replication stress (RS). RS refers to a variety of events of endogenous or exogenous origin that interfere with the progression of DNA replication forks. In cancer cells, RS is caused by the aberrant activation of oncogenes, which may either increase conflicts between replication and transcription or uncouple DNA synthesis from nucleotide metabolism.

RS activates a surveillance pathway known as the replication checkpoint. In this pathway, the ATR kinase is recruited to stressed forks by the accumulation of replication protein A (RPA)-coated single-stranded DNA and is activated by TopBP1, a factor loaded at single-stranded/double-stranded DNA junctions by the 9-1-1 complex (RAD1, RAD9, and HUS1) and its clamp loader, RFC3. Once activated, ATR phosphorylates the effector kinase CHK1 on Ser317 and Ser345 to amplify the checkpoint signal. This process is mediated by Claspin, Timeless, and Tipin, which form a complex at replication forks and act as mediators for CHK1 activation. Once activated, the ATR-CHK1 pathway acts in many ways to coordinate fork repair processes, prevent premature entry into mitosis and allow the completion of DNA replication.

Oncogene-induced RS is a double-edged sword. Although it contributes to cancer development by promoting genomic instability, it slows down cell proliferation and activates anticancer barriers leading to apoptosis or senescence. To proliferate, cancer cells must therefore bypass these barriers, while avoiding severe replicative defects that are incompatible with cell survival. It is generally believed that cells adapt to oncogene-induced RS by modulating the intensity of the ATR-CHK1 checkpoint response. Indeed, ATR and CHK1 haploinsufficiencies enhance oncogene-induced tumor formation, but a more severe depletion of ATR is synthetic lethal with oncogene overexpression.

Collectively, these data indicate that the ATR-CHK1 pathway has both protumoral and antitumoral activities depending on the cellular context. Understanding how cancer cells control this balance represents therefore a major challenge in cancer biology.

Owing to their central position in the ATR-CHK1 pathway and their fork association, Claspin, Timeless, and its partner Tipin are ideally placed to fine tune the cellular response to oncogene-induced RS. These factors are upregulated in many different cancers and their increased expression is associated with bad prognosis. Overexpression of Claspin is also a marker of radioresistance in metastasis lung cancer and Timeless is a candidate molecular marker for predicting the response of ER-positive postmenopausal breast cancer to Tamoxifen. However, the mechanism by which Claspin, Timeless, and Tipin promote cancer progression is currently unclear.

Besides their role in the ATR-CHK1 pathway, Claspin, Timeless, and Tipin also play a structural role at replication forks that is independent of their checkpoint function. Indeed, these three proteins form a complex at replication forks called the fork protection complex (FPC), which is conserved from yeast to vertebrates. Mrc1, Tof1, and Csm3, the budding yeast homologs of Claspin, Timeless, and Tipin, interact with DNA polymerase ε and the CMG helicase on the leading strand synthesis and are required for normal fork progression in a checkpoint-independent manner. In vertebrates, Claspin is a DNA binding protein that associates with branched structures in a highly specific and strong manner and interacts with numerous components of the replication machinery, namely MCM proteins, DNA polymerases (pol) α, δ, ε, CDC7 kinase, and CDC45. Timeless and Tipin interact with the replicative helicase components MCM2-7 and CDC45, and with replicative polymerases Pol ε and Pol δ, increasing their processivity in vitro. Since replication defect in Timeless-depleted cells is synthetic with ATR depletion, Timeless could coordinate enzymatic activities at the fork, independently of ATR.

Here, we have investigated the mechanism by which cancer cells adapt to oncogene-induced RS by modulating components of the ATR-CHK1 pathway. We have analyzed the expression of key components of this pathway in primary lung, breast, and colon cancer samples and in a variety of cancer cell lines. We found that unlike the checkpoint sensors ATR, RAD9, and RAD17, the downstream components of the ATR pathway Claspin, Timeless, and CHK1 show a correlated overexpression in cancer cells. To characterize the protumoral effect of this increased expression, we have reduced the excess of Claspin and Timeless in HCT116 cells under conditions that do not prevent checkpoint signaling. We show that this depletion reduces fork speed, increases fork stalling and leads to accumulation of γ-H2AX, in a checkpoint-independent manner. Moreover, primary fibroblasts escaping oncogene-induced senescence overexpress Claspin and Timeless. Altogether, these data indicate that cancer cells adapt to RS by overexpressing Claspin and Timeless, independently of ATR signaling.

Results

Components of the ATR pathway are overexpressed in cancers.

The ATR-CHK1 pathway controls tumor progression in a dosage-dependent manner, but the mechanism by which tumor cells modulate this pathway to adapt to oncogene-induced RS is currently unclear. To address this question, we have analyzed the expression of the checkpoint sensors RAD17, RAD9, and ATR, the mediators Claspin and Timeless and the effector CHK1 in 93 primary non–small-cell lung cancers (NSCLC), 74 primary colorectal carcinomas, and 206 primary breast cancers. For each gene, mRNA levels were determined by a quantitative real-time polymerase chain reaction (qRT-PCR) and were expressed relative to normal adjacent tissue (lung and colorectal cancers) or to a pool of healthy tissues (breast cancer). This analysis revealed that Claspin, Timeless, and CHK1 were overexpressed in these three cohorts in a correlated manner, with a Spearman coefficient ranging from 0.56 to 0.80. In contrast, the expression of the checkpoint sensors ATR, RAD9, and RAD17 was only modestly increased in cancer samples relative to normal tissues. A clustered expression of Claspin, Timeless, and CHK1 was also observed when a larger number of DNA damage response (DDR) genes were analyzed (Supplementary Fig. 1a–c), supporting the view that the downstream components of the ATR-CHK1 pathway are co-regulated.

In lung cancer, the expression of Claspin, Timeless, and CHK1 correlated also to some extent with PCNA, but this correlation was not observed in colon and breast cancers. In addition, PCNA mRNA levels were only moderately increased (1.4-fold) in lung cancer compared to those of Claspin and CHK1 (4.5- and 4.4-fold, respectively). The increased expression of Claspin, Timeless, and CHK1 in cancer cells does not simply reflect increased proliferation. Altogether, these data indicate that the upstream and downstream components of the ATR-CHK1 pathway are differentially regulated in primary cancer cells.
**Fig. 1** Claspin, Timeless, and CHK1 mRNA are overexpressed in primary colorectal, lung, and breast cancers. 

a. Schematic representation of the ATR pathway showing the six checkpoint factors analyzed in this study. 

b. Relative mRNA levels of ATR, RAD9, RAD17, Claspin, Timeless, CHK1, and PCNA in 93 lung tumors, 74 primary colorectal carcinomas, and 206 breast cancer samples. mRNA levels were determined by quantitative RT-PCR and are expressed as the ratio of tumor (T) to normal tissues (N). 

c. Nonparametric Spearman correlation analysis of the expression of checkpoint genes and proliferation marker (PCNA) in lung, colorectal, and breast cancer samples. 

d. High expression of Claspin, Timeless, and CHK1 is associated to bad prognosis in NSCLC patients. Disease-free survival (DFS) according to gene level expression: Claspin, Timeless, CHK1, RAD17, ATR, and RAD9. Totally, 72 patients were analyzed.
Claspin and Timeless are prognostic biomarkers of NSCLC. To determine whether the overexpression of the downstream components of the ATR-CHK1 pathway could have an impact on cancer development, we focused on a cohort of low grade (IA, IB, IIA, and IIB) NSCLCs patients who received no adjuvant treatment and asked whether the expression level of components of the ATR-CHK1 pathway determined at diagnosis had an impact on their disease-free survival (DFS) over a 72 months period. For each of these genes, patients were separated in two groups depending on the level of the corresponding mRNAs. Remarkably, the group of patients with tumors overexpressing Claspin, Timeless, or CHK1 showed a marked decrease in DFS compared to low-expression group (Fig. 1d). In contrast, the effect of RAD9, RAD17, and ATR levels was not significant (Fig. 1d). Together, these data indicate Claspin, Timeless, and CHK1 are frequently overexpressed in primary cancers independently of upstream components of the ATR-CHK1 pathway. In lung cancer, this increased expression was associated with a reduced DFS, suggesting that it could be used as a prognostic biomarker for early NSCLC. Antibodies against Claspin and Timeless have been successfully used on paraffin-embedded tumor samples25,26, which was confirmed here for Timeless with breast cancer samples (Supplementary Fig. 1a, b).

Claspin and Timeless are overexpressed in cancer cell lines. To determine how Claspin, Timeless, and CHK1 could promote tolerance to RS independently of ATR signaling, we first checked their protein levels in cancer cell lines and immortalized primary cells. Immunoblots confirmed that unlike ATR and RAD17, levels of Claspin, Timeless, and CHK1 were highly increased in transformed cells (U2OS, HeLa, and HCT116) compared to immortalized primary fibroblasts (IMR90, BJ), immortalized primary epithelial cells (RPE-1 and MCF10A) (Fig. 2a, b). Chromatin fractionation experiments also revealed that the amount of chromatin-bound Claspin and Timeless was proportionally increased in HCT116 cells (Supplementary Fig. 2), suggesting that a large fraction of overexpressed proteins was present on chromatin and could therefore play a biological role.

Next, we asked whether protein levels of Claspin, Timeless, and CHK1 are correlated in cancer cell lines. To this end, we monitored the relative abundance of 18 checkpoint proteins in the proteomic landscapes of 50 colorectal cancer cell lines43. This analysis confirmed that the abundance of Claspin, Timeless, and CHK1 is highly correlated and that these proteins form a cluster with BRCA1, TopBP1, and Tipin that is distinct from ATR, ATRIP, RAD9A, HUS1, and RAD1 (Fig. 2c). This is reminiscent of the correlation observed at the mRNA level in cancer patients (Supplementary Fig. 1d, f, h) and supports the view that Claspin, Timeless, and CHK1 are part of a functional module whose function is distinct from the ATR signaling pathway.

Excess of Claspin is dispensable for CHK1 activation. CHK1 plays pleiotropic roles in the cell and is essential for viability44-46. We, therefore, focused subsequent analyses on the function of Claspin and Timeless, using HCT116 colon cancer cells as an experimental model. To evaluate the functional significance of the overexpression of Claspin and Timeless in HCT116 cells, we first expressed shRNAs against these factors and observed a reduction in cell proliferation, growth on soft agar (Fig. 3a-c) and colony formation (Supplementary Fig. 3e) relative to control cells. Since the best-characterized function of Claspin and Timeless is to mediate checkpoint activation in S phase9,10, we next monitored the ability of sh-CLSPN and sh-TIM cells to promote CHK1 phosphorylation in response to nucleotide depletion by hydroxyurea (HU). Remarkably, CHK1 was efficiently phosphorylated on Ser 317 in sh-CLSPN and sh-TIM cells (Fig. 3d) and the CHK1 target CDC25A was rapidly degraded in a CHK1-dependent manner in HU-treated sh-CLSPN and sh-TIM cells (Fig. 3e). Similar results were also obtained with a lower dose of HU (100 μM; Supplementary Fig. 3a) or after a transient transfection with siRNAs against Claspin, Timeless, or both proteins (Supplementary Fig. 3b). These data indicate that the residual levels of Claspin and Timeless in sh-CLSPN and sh-TIM cells are sufficient for the timely activation of the ATR-CHK1 pathway in response to RS. Efficient CHK1 activation was also observed in U2OS and MCF7 cells transfected with siRNAs against Claspin and Timeless (Supplementary Fig. 3c, d), under conditions that interfere with the growth of MCF7 cells (Supplementary Fig. 3f). Altogether, these data indicate that the overexpression of Claspin and Timeless in cancer cell lines promotes cell growth independently of their checkpoint function.

High levels of Claspin and Timeless promote fork progression. In unchallenged growth conditions, the reduction of Claspin and Timeless levels induced the formation of γ-H2AX in HCT116 cells (Fig. 4c) and in U2OS and MCF7 cells (Supplementary Fig. 3c–d), which may reflect the induction of spontaneous RS. Since H2AX can also be phosphorylated by ATM and DNA-PK outside of S phase47, ongoing DNA replication was labeled with EdU to specifically monitor γ-H2AX levels in S-phase cells. Flow cytometry analyses revealed that 60% and 69% of EdU-positive cells were enriched in γ-H2AX upon reduction of Claspin or Timeless levels, respectively, whereas it is only the case in 22% of untreated HCT116 cells (Supplementary Fig. 4a). Immunofluorescence experiments confirmed that γ-H2AX levels in EdU-positive cells were also significantly higher in sh-CLSPN and sh-TIM cells relative to control cells (Fig. 4a, b). These data indicate that the shRNA-mediated reduction Claspin and Timeless levels in HCT116 cells increases spontaneous RS.

Next, we used DNA combing to monitor the impact of Claspin and Timeless levels on the progression of individual replication forks. To this end, sh-Ctrl, sh-CLSPN, and sh-TIM HCT116 cells were labeled for 10 min with EdU and for 20 min with CldU. DNA fibers were stretched on silanized coverslips and the length of CldU tracks was measured as described previously48. CldU tracks were significantly shorter in sh-CLSPN and sh-TIM cells than in sh-Ctrl cells (16.6 and 18.4 kb, respectively, vs. 22.1 kb in control cells, p < 0.001), indicating that replication forks are ~25% slower upon reduction of Claspin or Timeless levels (Fig. 4d). This slow fork progression was also confirmed with a related DNA fiber assay called DNA fiber spreading49, using inducible shRNAs (Supplementary Fig. 4b) or siRNAs (Supplementary Fig. 4c) against Claspin and Timeless or both (Supplementary Fig. 4c). To determine whether this slow fork phenotype is due to an increased rate of fork stalling, we measured the level of asymmetry between the distance covered by sister replication forks, as described earlier50. This analysis revealed a significant increase in fork asymmetry upon reduction of Claspin or Timeless levels in HCT116 cells (Supplementary Fig. 4d). Together, these data indicate that high levels of Claspin and Timeless in HC116 cells protect them from endogenous RS, independently of their ability to activate CHK1.

Claspin and Timeless promote genome stability in S phase. The budding yeast homologs of Claspin and Timeless, namely Mrc1 and Top1, promote replication fork progression through sequences that are intrinsically difficult to replicate, in a checkpoint-independent manner49,51,52. Common fragile sites (CFSs) are regions of the human that are replicated in late S phase and break more frequently under RS conditions53,54. To
determine whether late-replicating regions are particularly difficult to replicate in the absence of Claspin and Timeless, sh-Ctrl, sh-CLSPN, and sh-TIM HCT116 cells were sorted by flow cytometry and were collected in G1, early S, and late S phase (Fig. 4e). Immunoblotting of γ-H2AX in each subpopulation confirmed that γ-H2AX levels were globally higher during S phase in sh-CLSPN and sh-TIM cells compared to control cells and revealed that γ-H2AX was preferentially enriched at late-replicating regions in the absence of Timeless (Fig. 4f). We also monitored replication fork progression in the same samples by DNA combing. In sh-CLSPN cells, we observed an overall reduction of fork speed relative to control cells, regardless of the S-phase stage (Fig. 4g). In sh-TIM cells, this slowdown of the forks was only detected in late S phase, which is consistent with their increased level of γ-H2AX (Fig. 4f). Since CFSs replicate late during S phase, we next asked whether these loci break more frequently in the absence of Claspin and Timeless. To this end, we have analyzed copy-number variations (CNVs) and loss of heterozygosity in these cells by single-nucleotide polymorphism comparative genomic hybridization (SNP-CGH). This analysis revealed 33 recurrent rearrangements in sh-Ctrl cells (Supplementary Fig. 4e), which are typical of HCT116 cells. In addition, 14 specific

Fig. 2 Coordinated upregulation of Claspin, Timeless, and CHK1 protein levels in cancer cell lines. a Western blot analysis of the levels of Claspin, Timeless, CHK1, and ATR in human cancer cell lines (U2OS, HeLa, and HCT116) and in immortalized primary fibroblasts (IMR90). b Claspin, Timeless, CHK1, RAD17, and ATR in colorectal cancer HCT116 cells, immortalized primary fibroblasts (BJ) and immortalized epithelial cells (RPE-1 and MCF10A). c Correlation analysis of the abundance of DDR proteins in 50 colorectal cancer cell lines.
Fig. 3 The overexpression of Claspin and Timeless promotes cell growth in a checkpoint-independent manner. a Levels of Claspin, Timeless, and CHK1 in immortalized IMR90 fibroblasts and in HCT116 cells transduced with a control shRNA (sh-Ctrl) or with shRNAs against Claspin (sh-CLSPN) and Timeless (sh-TIM). b Growth of HCT116 transduced with doxycycline-inducible shRNAs against Claspin (sh*-CLSPN) or Timeless (sh*-TIM) or with a control shRNA (sh*-Ctrl). Cell growth was determined 48 h after addition of doxycyclin. **p < 0.05. c Anchorage-independent growth assay. HCT116 cells transduced with sh*-Ctrl, sh*-CLSPN and sh*-TIM inducible shRNAs were grown 2 weeks in soft agar medium (0.7%) in the absence (−Dox) or the presence (+Dox) of Doxycyclin. Colonies were visualized by microscopy and mean colony size was determined with MetaMorph. Representative images are shown. d Western blot analysis of CHK1 phosphorylation (p-CHK1 and S317) in asynchronous sh-Ctrl, sh-CLSPN, and sh-TIM HCT116 cells treated for 0, 1, or 6 h with 10 mM HU. e Checkpoint-dependent degradation of CDC25A in sh-Ctrl, sh-CLSPN, and sh-TIM HCT116 cells treated for 0 or 6 h with 10 mM HU and 300 nM of the CHK1 inhibitor UCN01.
rearrangements were detected in sh-CLSPN cells and 22 in sh-TIM cells, almost half of which colocalized with CFSs (Supplementary Fig. 4e, f). Collectively, these data indicate that increased levels of Claspin and Timeless promote replication fork progression and prevent genomic instability in regions of the genome that are intrinsically difficult to replicate, such as CFSs.

**Claspin and Timeless protect cells from oncogenic RS.** Spontaneous DNA repair foci are frequently detected at early stages of tumorigenesis as a consequence of oncogene-induced RS, but generally disappear at later stages, which suggests that cancer cells adapt to RS. To determine whether the overexpression of Claspin and Timeless helps cells tolerate oncogene-induced RS, an oncogenic form of Ras (RasV12) was expressed in immortalized BJ-hTERT fibroblasts with a doxycyclin-inducible promoter. As expected, RasV12 increased the levels of phospho-CHK1 and γH2AX (Supplementary Fig. 5a) and induced replication fork progression defects (Supplementary Fig. 5b) as reported by others. We also observed an induction of p16 expression (Supplementary Fig. 5c), indicating that cells have undergone oncogene-induced senescence (OIS).
After 60 days of RasV12 expression, a population of cells escaping senescence was detected which is consistent with earlier studies. These cells showed no detectable levels of p16 (Supplementary Fig. 5c), but were unable to form colonies, suggesting that they were not tumorigenic.

Next, we isolated thirteen individual clones from the population of OIS-resistant BJ-RasV12 cells and the expression of ATR, Claspin, Timeless, and CHK1 was analyzed by qRT-PCR. Remarkably, the majority of these clones showed at least a 50% increase in the levels of Claspin and CHK1 mRNAs compared to BJ-hTERT, whereas ATR levels remained unchanged (Fig. 5a). Among them, the clones #4 and #5 overexpressing both Claspin and Timeless were selected for further analyses, together with clone #8, in which these factors were not overexpressed. Western blot analyses confirmed that clones #4 and #5 (but not clone #8) contained increased protein levels of Claspin, Timeless, and CHK1, whereas levels of ATR, besides Claspin and Timeless.

However, DDR signals decrease during cancer development, as illustrated in bladder cancer. Recent evidence indicates that this decrease of DDR signaling correlates with increased expression of DNA repair genes (http://repairtoire.genesilico.pl). Among these, only DNA repair genes allowed a good separation between the three clones (Fig. 5c). Thus, we have performed a differential expression analysis using a SAM multiclass analysis focusing on different sets of genes induced by E2F or E2F hyperactivation (siE2F6), HU exposure, and genes involved in DNA repair (http://repairtoire.genesilico.pl). Among these, only DNA repair genes allowed a good separation between clone #4 and #5 and BJ or BJ-Ras cells, clone #8 showing intermediate profiles (Supplementary Fig. 5d). These data suggest that overexpressed factors contribute to RS tolerance besides Claspin and Timeless.

Since clone #4 and #5 overexpress a large number of replication factors besides Claspin and Timeless (Fig. 5f), Fold change > 1.5; FDR = 0), we next asked whether depletion of only Claspin or Timeless would be sufficient to prevent RS tolerance, as shown above for HCT116 cells (Fig. 4d). To address this possibility, clone #4 and #5 were transfected with siRNA pools against Claspin and Timeless (Fig. 6a) and fork progression was monitored by DNA fiber spreading. This analysis confirmed the slow fork phenotype induced by the expression of RasV12 in BJ cells and the ability of clones #4 and #5 (but not #8) to restore a normal fork progression despite the induction of RasV12 (Fig. 6b).

Remarkably, this adaptation was lost upon depletion of Claspin or Timeless in clones #4 and #5, as manifested by slower fork progression (Fig. 6b) and increased γ-H2AX levels (Supplementary Fig. 6a). These data suggest that the overexpression of Claspin and Timeless is a spontaneous event that is selected at early stages of the cancer process to protect cells from chronic RS.

To determine whether the overexpression of Claspin or Timeless is not only necessary but also sufficient to promote resistance to oncogene-induced RS, these proteins were expressed in BJ-RasV12 (Supplementary Fig. 6b) and in U2OS-CycE cells (Fig. 6c) and their ability to restore normal fork progression was monitored by DNA fiber spreading after induction of RasV12 in BJ-hTERT cells or CycE in U2OS cells. In BJ-RasV12 cells, the overexpression of Claspin, Timeless, or both proteins rescued fork slowdown induced by RasV12 in BJ-hTERT cells (Supplementary Fig. 6c) and by CycE in U2OS cells (Fig. 6d). Together, these data indicate that increased levels of Claspin or Timeless is sufficient to protect cells from oncogene-induced RS.

Discussion

Oncogene-induced RS plays an active role in tumorigenesis by promoting genomic instability and by inducing a selective pressure for the inactivation of DDR factors such as TP53 and ATM. Spontaneous DNA damage and genomic instability are caused by DNA replication problems occurring in pretumoral lesions. However, DDR signals decrease during cancer development, as illustrated in bladder cancer. Recent evidence indicates that this decreased DDR signaling correlates with increased expression of the checkpoint mediator Timeless in bladder tumors. However, the mechanism by which Timeless promotes adaptation to oncogene-induced RS is currently unclear.

Here, we have monitored the expression of Claspin and Timeless in primary breast, colorectal, and lung cancers and compared it to other components of the ATR-CHK1 pathway. This analysis revealed that Claspin, Timeless, and CHK1 are overexpressed in a coordinated manner in these three cancers whereas the checkpoint sensors ATM, RAD17, and RAD9 are not. We confirmed that Claspin and Timeless protein levels are much higher than those of CHK1 in these cancers.
higher in cancer cell lines relative to immortalized fibroblasts, which is consistent with earlier studies. Moreover, we observed a tight correlation between the abundance of Claspin, Timeless, and CHK1 proteins in the proteomic landscape of 50 colon cancer cell lines. Together, these data indicate that the downstream components of the ATR-CHK1 pathway define a functional module that is specifically enhanced in cancer cells.

Importantly, we also found that in low-grade NSCLC, increased expression of Claspin, Timeless, and CHK1 (but not ATR, RAD9, and RAD17), correlates with the aggressiveness of...
the tumor. This finding is important because surgical resection is the standard of care for NSCLC and recurrence after resection has been reported in 30–75% of all cases from stage I to stage III⁶⁵. The current clinical staging based on anatomic or pathological factors is insufficient to predict the evolution of the disease, especially for early stage diseases. Our data indicate that Claspin and Timeless could be used as prognostic markers to identify patients who would benefit from adjuvant chemotherapy.

Claspin and Timeless have a dual role in checkpoint signaling and in the maintenance of replication fork integrity ³⁴–³⁶. To determine which of these two activities promotes RS tolerance, we have reduced the expression of Claspin and Timeless to levels that impede cell growth without interfering with ATR-CHK1 signaling. Conflicting results have been published regarding the impact of Claspin and Timeless depletion on CHK1 activation ³³,⁷⁰. Our data indicate that residual levels of Claspin and Timeless are sufficient to activate CHK1. Alternatively, other checkpoint mediators such as TopBP1 and BRCA1 could be redundant with Claspin and Timeless ³³,⁷⁰. Our data indicate that the abundance of TopBP1 and BRCA1 correlates with Claspin, Timeless, and CHK1 in colon cancer cell lines, which would be consistent with the latter hypothesis. In any case, we report here that the reduction of Claspin and Timeless levels in cancer cell lines reduced the speed of fork progression, increased the rate of fork stalling and increased γ-H2AX levels in checkpoint-proficient cells. We, therefore, propose that enhanced levels of Claspin and Timeless protect cancer cells from endogenous RS in a checkpoint-independent manner.

The mechanism by which an excess of Claspin and Timeless promote RS tolerance is currently unclear. A large body of

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Fig. 6 Increased levels of Claspin and Timeless promote fork progression in cells expressing oncogenic Ras. a Protein levels of Claspin and Timeless in clones #4 and #5 transfected with control (si-Ctrl), Claspin (si-CLSPN), and Timeless (si-TIM) siRNAs. b DNA fiber spreading analysis of fork progression in clones #4 and #5 after depletion of Claspin and Timeless with siRNAs. Median CldU tracks length (red) were determined for three independent experiments. ****p < 0.0001, ***p < 0.001, ns nonsignificant difference. Mann-Whitney rank sum test. c Levels of Claspin and Timeless in U2OS-CycE cells transfected with GFP, Claspin, and Timeless plasmids. U2OS-CycE cells expressing Cyclin E under the control of a doxycyclin-inducible promoter were grown during 6 days in the presence of Dox to induce replication stress. Then cells are transfected with plasmids to overexpress GFP, Claspin or Timeless during 3 days before performing DNA spreading experiment. d DNA spreading analysis of experiment performed in c. DNA spreading was also performed on U2OS-CycE cells growing without Dox. Median CldU tracks length (red) were determined for three independent experiments. ****p < 0.0001, ns nonsignificant. Mann-Whitney rank sum test.
Moreover, they represent valuable prognostic markers for data indicate that Claspin and Timeless represent promising response, which would be detrimental to tumor growth. These fork progression without triggering an increased checkpoint to cancer cells experiencing chronic RS by promoting replication expression of Claspin and Timeless protects forks from chronic overexpression of a large group of DDR genes involved in fork with a simple upregulation of the E2F pathway, but with a global heterogeneous gene-expression pro cancer cell lines. As in cancer cell lines, the depletion of Claspin also detected increased levels of CHK1 mRNAs, but not ATR, increased levels of Claspin and to a lesser extent Timeless. We OIS12,13. Remarkably, a large fraction of these clones showed enhanced levels of Claspin, Timeless, and Tipin in cancer cells may promote the reassembly of the FPC and accelerate fork restart. This is consistent with the fact that overexpressed Claspin and Timeless accumulate on chromatin in HCT116 cells and could be easily available to reform the FPC upon stress-induced dissociation. Interestingly, the overexpression of Timeless in bladder cancer is associated with increased genomic instability25. It is, therefore, likely that the adaptation to oncogene-induced RS mediated by enhanced Claspin and Timeless occurs at the expense of genome integrity and would therefore promote cancer progression.

Another important question raised by our study concerns the mechanism by which cancer cells overexpress Claspin and Timeless. The expression of Claspin and CHK1 depends on the E2F pathway61,75, which are upregulated by oncogenes and contributes to RS tolerance61. To determine whether the upregulation of the E2F pathway is the initial event contributing to the upregulation of Claspin and Timeless, we have overexpressed an oncogenic form of Ras in immortalized primary fibroblasts and monitored the expression of Claspin, Timeless in clones escaping OIS12,13. Remarkably, a large fraction of these clones showed increased levels of Clasin and to a lesser extent Timeless. We also detected increased levels of CHK1 mRNAs, but not ATR, which is consistent with the levels observed in tumor samples and cancer cell lines. As in cancer cell lines, the depletion of Claspin and Timeless in these clones increased oncogene-induced RS, stressing the importance of these proteins the adaptation to stress. Transcriptome analyses of these clones revealed complex and heterogeneous gene-expression profiles that are not consistent with a simple upregulation of the E2F pathway, but with a global overexpression of a large group of DDR genes involved in fork protection. Interestingly, one of the OIS-resistant clones did not overexpress Claspin and Timeless, indicating that alternative pathways exists to adapt to RS.

In conclusion, our work supports a model in which the overexpression of Claspin and Timeless protects forks from chronic RS by stabilizing the replisome without increasing the activation of the ATR pathway. This would confer a proliferative advantage to cancer cells experiencing chronic RS by promoting replication fork progression without triggering an increased checkpoint response, which would be detrimental to tumor growth. These data indicate that Claspin and Timeless represent promising targets for anticancer therapies targeting replication forks. Moreover, they represent valuable prognostic markers for patients with early NSCLC.
spreading were performed as described48,49,77 using the following combination of CNVs and estimate copy number.

were analyzed with the Miltenyi Macs Quant analyzer and FlowJo software at the body (GE Healthcare) and visualized using the ECL-chemiluminescence method (Pierce).

Cell fractionation. Cells were lysed with 0.2% Triton X-100 CSK buffer (10 mM PIPES [pH 6.8], 100 mM NaCl, 500 mM sucrose, MgCl2, 1 mM EGTA, 1 mM EDTA, protease inhibitors cocktail (Complete, EDTA-free tabletic Roche), and 0.2% Triton X-100 for 10 min on ice. After centrifugation (0.8 g for 5 min), the supernatant is the soluble fraction (sup). The pellet was washed once more with the same buffer and incubated 10 min on ice. After centrifugation (0.8 g for 5 min) the pellet (chromatin fraction) was resuspended in 2x Laemmli buffer.

Cell proliferation quantification. The Cell Proliferation Reagent WST-1 (Sigma) is used for the spectrophotometric quantification of cell proliferation.

Soft agar assay. Totally, 5 × 104 cells were resuspended in 3 ml of prewarmed DMEM-10% FBS and were mixed with 3 ml of 0.8% agar solution (Sigma A5431) in 10% DMEM and 1% FBS. A total of 1.5 ml of cell suspension is poured in three wells containing a 2 ml prewarmed layer of 1% agar and 1% DMEM and 1% FBS. Totally, 1 ml of medium is added on top of agarose layer and plates are incubated for 15 days at 37°C. Top medium is replaced every three days. Colonies were visualized with a microscope and colony sizes were determined with the Meta Morph software.

Colony formation assay. HCT116 cells are plated in 6-well plates at 20% confluence and are grown for 2 weeks. Cells are washed twice with ice-cold phosphate-buffered saline (PBS) and fixed for 10 min with ice-cold methanol. Colonies are stained for 10 min at room temperature with 0.5% crystal violet in 25% methanol and rinsed with ddH2O.

Cell sorting. After trypsinization, asynchronous cells were resuspended in PBS-3 mM EDTA (106 cells/500 µl cells) and fixed in ETOH 70% as in the previous section. After two washes in ice-cold PBS, cells were resuspended at the concentration of 5 × 105 cells/ml in staining buffer (3 mM EDTA, 0.05% NP40, 50 µg/ml propidium iodide, 1 mg/ml RNase A in PBS). Cells in G1, early S, and late S phase were collected according to their DNA content with a FACS-Aria cell sorter (Becton Dickinson) at the IGM-MIRI facility (Montpellier, France). Immediately after collection, each fraction of cells was divided in two parts: one for proteins analysis by western blot and one for DNA combing.

Flow cytometry analysis of γH2AX and EdU. Asynchronous cells were pulse labeled with 10 µM EdU (5-ethyl-2′-deoxyuridine) for 30 min before harvesting. Cells were trypsinized as usual, washed with cold PBS and once with PBS–bovine serum albumin (BSA) 1%. A total of 1 × 106 cells were fixed in 2% formaldehyde–PBS for 10 min in the dark at room temperature and washed twice in 1 ml of cold PBS/1% BSA. Cells were permeabilized by addition of 200 µl of saponin buffer (0.1% saponin in PBS/0.5% BSA) for 10 min at 4°C and incubated with γH2AX antibody (Millipore 05-636) diluted at 1/500 in PBS. Cells in G1, early S, and late S phase were collected according to their DNA content with a FACS-Aria cell sorter (Becton Dickinson) at the IGM-MIRI facility (Montpellier, France). Immediately after collection, each fraction of cells was divided in two parts: one for proteins analysis by western blot and one for DNA combing.

DNA combing and DNA fiber spreading. DNA combing and DNA fiber spreading were performed as described44,45,47 using the following combination of antibodies. Primary antibody mix: Mouse anti-BrdU to detect IdU (1/100 PBS/T, BD 347580), Rat anti-BrdU to detect CldU (1/100 PBS/T, Eurobio clone BU175), Anti-siDNA (1/100 PBS/T, auto anti-siDNA, DSHB). Secondary antibody mix: Goat anti-rat Alexa 488 (1/100 PBS/T, Molecular Probes, A11080), Goat anti-mouse IgG(Alexa 546 (1/100 PBS/T, Molecular Probes, A21123), Goat anti-Mouse IgG2a Alexa 647 (1/50 PBS/T, Molecular Probes, A21241). Statistical analysis of track lengths is performed with GraphPad Prism 7.0.

Immunofluorescence. HCT116 cells were seeded on cover glasses and labeled with 10 mM EdU for 15 min. Cells were then fixed with 2% paraformaldehyde and permeabilized using 0.1% Triton X-100. Immunodetection of γH2AX in S-phase was performed overnight at 4°C using a specific antibody (1/100; 05-636, Millipore). Click-it chemistry was performed according to instructions. Images were acquired using an LSM780 Zeiss confocal microscope. Mean fluorescence intensity of γH2AX in EdU-positive cells was quantified with CellProfiler.

Transcriptome analysis. RNA sequencing (RNA-seq) libraries were prepared using the Illumina TruSeq Stranded mRNA Library Prep Kit. Paired-end RNA-seq were performed with an Illumina NextSeq sequencing instrument (Helixio, France). RNA-seq read pairs were mapped to the reference human GRCh37 genome using the STAR aligner35. Statistical analyses were performed with R (v3.2.3) and R packages developed by BioConductor project. The expression level of each gene was summarized and normalized using DESeq R/Bioconductor package36. Differential expression analysis was performed using SAM multiclass analysis (samm package). Genes with a fold change ≥1.5 and a FDR <0.05 were considered as significantly differentially expressed.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. The RNA-seq datasets generated and analyzed during the current study (Fig. 5f and Supplementary Fig. 5d) are available in the GEO repository, accession number: GSE123380. A reporting summary for this Article is available as a Supplementary Information file.

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Author contributions

J.N.B., V.B., M.-J.P., Y.-L.L., A.-L.S. and H.T. performed the experiments. J.G., A.L. and J.M. analyzed transcriptomic data and clinical data. M.L.T. performed immunochemistry experiments. J.M. analyzed transcriptomic data. T.I.R. and J.C. perform proteomic analysis. J.-S.H., H.T. and P.P. designed the experiments. H.T. and P.P. wrote the manuscript.

Additional information

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