Transcriptional Regulation of the P-bodies Associated Psoriasis Candidate Gene *CCHCR1*

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ABSTRACT

CCHCR1 (coiled-coil alpha-helical rod protein 1) is a candidate gene of psoriasis and was recently identified as a novel component in P-bodies, the site for regulating mRNA turnover, and a protein in centrosomes. Little is known about its transcriptional regulation or its functions in cell cycle progression. In this study, *CCHCR1* was found in a close (287-bp) head-to-head orientation with its neighboring gene, *TCF19*. The transcription of the *CCHCR1/TCF19* gene pair was controlled by a shared bidirectional promoter and was induced at the G1/S transition of the cell cycle. The 287-bp intergenic sequence was sufficient for the G1/S expression of both genes, but the expression of *CCHCR1* was further promoted by the presence of exon 1 of *TCF19* and *CCHCR1*. The expression of the *CCHCR1/TCF19* gene pair was dependent on the E2F1 transcription factor. E2F1 binding sites were predicted in the *CCHCR1/TCF19* bidirectional promoter by *in silico* analysis. Overexpression of E2F1 induced the expression of *CCHCR1* and *TCF19*. In addition, E2F1 knockdown inhibited both *CCHCR1* and *TCF19* expression. Knockdown of *CCHCR1* or *TCF19* reduced cell count but only depletion of *CCHCR1* significantly induced *p21* expression, implying that *CCHCR1* and *TCF19* may both regulate cell growth but in divergent pathways. Taken together, we revealed a bidirectional regulation of the *CCHCR1/TCF19* gene pair in the G1/S transition and provide a new perspective to understand the role of *CCHCR1* as a candidate gene of psoriasis.

Introduction

CCHCR1 (coiled-coil alpha-helical rod protein 1) is a candidate gene of psoriasis, a common skin disease affecting 1% to 2% of the population[1]. CCHCR1 protein was found to be involved in multiple cellular processes, including regulation of keratinocyte proliferation and differentiation[2,3], steroidogenesis in mitochondria[4,5], myogenic differentiation[6], and cytoskeleton organization[7]. By using co-immunoprecipitation (co-IP) of CCHCR1 followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS), our lab found that CCHCR1 interacted with proteins in P-bodies and centrosomes, and the localization of CCHCR1 in these two cell compartments was confirmed with fluorescence microscopy[8]. RNA sequencing of CCHCR1-overexpressing cells supported a role of this protein in the P-bodies and centrosomes related pathways[9]. In the present study, we aimed to decipher the transcriptional control of the *CCHCR1* gene to gain further insight into its functions in cell proliferation and the cell cycle using HeLa cells as a model.

Psoriatic skin is characterized by hyper-proliferation and abnormal differentiation of the keratinocytes[10]. Many chemicals and hormones that regulate the proliferation of keratinocytes are reported to perturb the mRNA expression of *CCHCR1*. For example, insulin and estrogen, which promote the growth of keratinocytes, up-regulate *CCHCR1* expression[5]; Immunosuppressant cyclosporine A inhibits the proliferation of keratinocytes and down-regulates *CCHCR1*[5]. Neuronal cells are protected from cell division by the Rb pathway and inactivation of the Rb pathway resulted in cell cycle re-entry and up-regulation of *CCHCR1*[11]. Nonetheless, the expression of *CCHCR1* is not always positively correlated with active cell proliferation. Serum starvation followed by re-feeding of cultured immortalized human HaCaT keratinocytes down-regulates *CCHCR1*[12]. Anti-cancer drug camptothecin (CPT), which causes DNA damage, growth arrest and cell apoptosis[13-16], up-regulates *CCHCR1*[17]. Overexpression of CCHCR1 reduces keratinocytes proliferation[18]. Surprisingly, *CCHCR1* mRNA is down-regulated in cultured non-lesional psoriatic keratinocytes. It is suggested that down-regulation of *CCHCR1* in basal keratinocytes may enhance cell proliferation in the epidermis, which is relevant for the pathogenesis of psoriasis[5]. To address the discrepancy in the relation between *CCHCR1* expression and cell proliferation, it is important decipher the transcriptional control of *CCHCR1* throughout the cell cycle.

CCHCR1 is located in the human leukocyte antigen (HLA) region on chromosome 6. We noticed that the genome position of *CCHCR1* is very close to a gene, *TCF19* (transcription factor 19). *CCHCR1* has a head-to-head orientation with *TCF19* and their transcription start sites are only separated by 287 bp, raising a possibility that both genes are co-regulated by a single bidirectional promoter. *TCF19*, originally named as *SC1*, is predicted as a transcription factor due to the presence of a putative trans-activating domain. The function of *TCF19* is still vague, but it is known as a late growth-regulated gene in which its mRNA level is induced several hours later after mitogenic stimulation, and is maximally expressed in the G1/S transition or S phase of the cell cycle[19]. It has also been reported in association with pancreatic islet proliferation[20,21], HBV-related chronic hepatitis B, cirrhosis, hepatocellular carcinoma[22] and non-small cell lung cancer[23]. In this study, we revealed that *CCHCR1* and *TCF19* shared a bidirectional promoter which is activated in the G1/S transition of the cell cycle. We also identified E2F1, a transcription factor regulates the expression of genes involved in G1/S cell cycle transition and DNA synthesis[24], activated the *CCHCR1/TCF19* gene pair.

Results

Genome arrangement of *CCHCR1* gene indicates a possible shared bidirectional promoter with *TCF19*

The *CCHCR1* gene is located on the human chromosome 6p21.3, with a head-to-head orientation with its neighboring gene *TCF19*. In known bidirectional promoters, gene pair is usually separated by less than 1000 bp[25]. As the transcriptional start sites of *CCHCR1* and *TCF19* are separated by only 287 bp (Fig. 1a), we predicted that both genes use a shared bidirectional promoter. Analyzing nucleotide sequence just before the start codon of each gene, i.e., -1231 to +638 (Fig 1b; relative to the transcription start site of *CCHCR1*; NCBI Reference Sequence: NM_019052.3), revealed many characteristics of a classical bidirectional promoter. First of all, the region is TATA-less and consists of a CpG island (Fig 1B; -598 to +128) that overlapped the first exon of each gene[25]. In addition, this region contains many putative transcription factor binding sites such as GABP, MYC, E2F1, E2F4, CCAAT and YY1 (Supplementary Table 1 and 2), which are commonly over-represented in the sequence of the bidirectional promoter[26]. The highly enriched E2F transcription factor binding sites (Supplementary Table 2; 21 putative sites as predicted by bioinformatics tool MatInspector[27]) are a prominent feature in this region. By the Genevestigator software with the "Perturbations" microarray database, in which comprises responses to various experimental conditions (drugs, chemicals, hormones, etc.), diseases, and genotypes[28], we found that the expression of *CCHCR1* was positively correlated with many genes involved in G1/S transition or S cell cycle phase, including *E2F1* (a member of the E2F family) and *TCF19* (Supplementary Table 3). In addition, *CCHCR1* expression was also positively correlated with *TCF19* when being analyzed with the "Cell lines" and "Cancers" microarray database, indicating that these two genes have similar expression level in many different cell types (Supplementary Table 3). Based on all of the above observations, we speculate that *CCHCR1* may share a bidirectional promoter with *TCF19*, and both may be co-regulated by E2F1 in the cell cycle.

E2F1 induces the expression of CCHCR1 and TCF19

The E2F transcription factor family has 8 members: E2F1–3 are activators and E2F4–8 are repressors. To address the activity of E2F on the expression of *CCHCR1* and *TCF19*, we measured the mRNA level of *CCHCR1* and *TCF19* after overexpression of E2F1, 2 or 3 in HeLa cells. Among these three E2F activators, only the overexpression of E2F1 induced *CCHCR1* and *TCF19* expression (Fig. 2a). Knockdown of *E2F1* by siRNA (48 h post-transfection) significantly down-regulated the expression of *CCHCR1* and *TCF19* (Fig. 2b).

CCHCR1 and *TCF19* are up-regulated in G1/S transition

Because E2F1 regulated genes in the G1/S transition[29], we wondered whether *CCHCR1* and *TCF19* were co-regulated in the G1/S transition. HeLa cells were synchronized at the G1/S transition by a double thymidine block and then released to allow cell cycle progression (Fig. 3a and b). The expression patterns of *CCHCR1* and *TCF19* were highly associated (Fig. 3c). Their expression is the highest in the G1/S transition, remains high in S phase, and then decreased during the progression from the S phase to the G2/M phase, and the levels remained relatively low when progressing to the G1 phase (Fig. 3c). Although it is common that the cells start losing synchrony in another round of cell cycle, i.e., from 16 h to 20 h (Fig. 3a), we still observed a co-up-regulation of *CCHCR1* and *TCF19* when more cells were approaching S phase (Fig. 3c).

To confirm that the G1/S up-regulation of *CCHCR1* and *TCF19* is transcriptionally regulated by the bidirectional promoter instead of other confounding events, such as a change of mRNA stability, we cloned several promoter fragments into firefly luciferase (Fluc) reporter constructs to study their transcriptional activities during cell cycle progression. Fragments are either inserted in the forward direction or reverse direction with respect to the coding region of Fluc (Fig. 4a). The 287-bp fragment contains the *CCHCR1/TCF19* intergenic sequence, and the 556-bp fragment contains the intergenic sequence plus exon 1 of both genes (Fig. 4a). Analysis of these promoter fragments throughout the cell cycle revealed high activities in the G1/S transition and S phase, consistent with the cell cycle expression patterns of *CCHCR1* and *TCF19* (Fig. 3c and 4b). In addition, significantly higher activity of the *CCHCR1* promoter was observed in all cycle cell phases when exon 1 of both genes were included (Fig. 4a and b; $P_{CCHCR1}(556)$). As for *TCF19* promoter, similar activities were observed with or without exon 1 of both genes (Fig. 4b).

***CCHCR1* or *TCF19* knockdown reduces cell count**

CCHCR1 or *TCF19* was knockdown by siRNA and a decrease in cell number was observed 4 days after siRNA transfection, indicating either slower growth or an increase in cell death (Fig. 5a). As there are no good commercial antibodies to confirm the depletion of CCHCR1 and TCF19 proteins, we considered the decrease in the cell counts to be indirect proof of successful knockdown. Intriguingly, the expression of the cell cycle regulator *p21* was significantly increased in *CCHCR1* down-regulated cells (Fig. 5b).

***CCHCR1* and *TCF19* are induced by anticancer drug CPT**

We next extended our investigation on drugs that affects *CCHCR1* expression. The NCBI Gene Expression Omnibus (GEO) database indicated that the anticancer drug camptothecin (CPT) up-regulates the expression of *CCHCR1*[17]. CPT is a topoisomerase I inhibitor which induces cell cycle arrest at the G2 and S phase, and ultimately apoptosis[13-16]. As *CCHCR1* and *TCF19* are co-regulated in many experimental perturbation conditions (Supplementary Table 3), we expect to see a similar induction of *TCF19* upon CPT treatment. After 24 h treatment of CPT, the majority of the cells were arrested in S phase, and there was a minor accumulation of G2/M phase cells (Fig. 6a). Importantly, both *CCHCR1* and *TCF19* were up-regulated upon CPT treatment (Fig. 6b).

Discussion

The *CCHCR1* gene was first described as putative gene 8 (PG8), located in the centromeric end of the HLA class I region in chromosome 6[30]. It was once named *HCR* (α-**H**elix **C**oiled-Coil **R**od homolog) as its gene product was predicted to have an α-helical coiled-coil rod domain[31], and it is now officially called *CCHCR1*. Its altered expression in psoriatic skin[5] has received ample attention in clinical research in the last decade, but controversial results were found in linkage and association studies for *CCHCR1* as the psoriasis susceptibility gene, possibly due to the small sample sizes, different populations and different statistical analyses being used[32-34]. The studies concerning psoriasis candidate genes have been mainly genetic studies. As psoriasis is a complex multifactorial disease[35], it's no wonder that results are controversial. The current opinion concerning multifactorial diseases like psoriasis is that one risk allele cannot fully explain the pathogenesis. *CCHCR1* is a psoriasis susceptibility gene among others[36]. CCHCR1 expression has been linked to keratinocyte proliferation. However, results are contradictory among the studies. CCHCR1 is suggested as both a positive and negative regulator of cell proliferation. Insulin, estrogen, and EGF stimulated *CCHCR1* expression and keratinocyte proliferation[5]. Immunosuppressant cyclosporine A down-regulated *CCHCR1* and inhibited keratinocyte proliferation[5]. In contrast, cyclosporine A, T lymphocyte cytokine and IFN-γ, which stimulated the growth of psoriatic keratinocytes, down-regulated the expression of *CCHCR1*[37]. Confluent HaCaT (a human keratinocyte cell line) cell culture expressed a higher level of *CCHCR1* than sub-confluent proliferating HaCaT cells[12]. In addition, immunostaining of psoriasis lesions suggested that expression of CCHCR1 protein is inversely correlated with Ki-67 expression, a marker of cell proliferation[12,37]. To explain these contradictory findings of *CCHCR1* expression with regards to cell proliferation, we examined the expression of *CCHCR1* throughout the cell cycle progression and found that it is induced in the G1/S transition. *CCHCR1* expression level remained high in S phase and decreased when approaching the G2/M and G1 phase (Fig. 3c). We proposed that the *CCHCR1* expression is related to the cell cycle status within the cell population in addition to general cell proliferation.

Human genome sequencing revealed an abundance (>10%) of divergently transcribed gene pairs whose transcription start sites are separated by less than 1000 bp[38]. A shared part of intergenic fragment initiates and regulates transcription of the paired gene in both directions, thus forming a bidirectional promoter[38]. These genes are highly co-expressed[39] and may function in the same biological pathway such as DNA repair, cell cycle process, chromatin modification, metabolic process, and cell proliferation and differentiation[38,40]. There are multiple bidirectional promoters in human chromosome 6[41], on which *CCHCR1* and *TCF19* are located (Fig. 1b). Importantly, the genome arrangement of *CCHCR1* and *TCF19* is also conserved in mouse (head-to-head orientation with transcription start sites separated by 291 bp). Our data showed that *CCHCR1* and *TCF19* are co-regulated throughout the cell cycle (Fig. 3). Luciferase activity assay confirmed that *CCHCR1* and *TCF19* share a common bidirectional promoter for transcription regulation with maximal activation in the G1/S transition of the cell cycle and lowest activation in the G2/M and G1 phase (Fig. 4). In addition, we found that for optimal expression, *CCHCR1*, but not *TCF19*, required exon 1 of both *CCHCR1* and *TCF19* in addition to the intergenic region, revealing a potential enhancing element lies within the exon 1 regions that acting on the direction of *CCHCR1* transcription.

More than 20 putative E2F binding sites (Fig. 1b and Supplementary Table 2) were predicted in the *CCHCR1/TCF19* bidirectional promoter. All the eight members of the E2F family are transcription factors which play pivotal roles in controlling cell cycle progression, proliferation, differentiation and DNA damage checkpoints[29]. E2F1 is the first cloned member of the E2F family. E2F1 controls G1 to S phase transition and regulates cell cycle-dependent gene expression

through the CDK-Rb-E2F pathway, and growth inhibition and the apoptosis through the p53-p21-E2F pathway[42-45]. Our results suggested that E2F1 is involved in controlling the G1/S expression of the *CCHCR1/TCF19* gene pair (Fig. 2), potentially by binding to the *CCHCR1/TCF19* bidirectional promoter. However, we cannot rule out that E2F1 could induce the expression of *CCHCR1* and *TCF19* genes indirectly by controlling the activities of other transcription regulators of the *CCHCR1/TCF19* bidirectional promoter. E2F1 requires the cooperation of other transcription factors such as CAAT or YY1 to mediate its cell cycle-specific activity[46]. Interestingly, we identified putative CAAT and YY1 binding sites within the *CCHCR1/TCF19* bidirectional promoter (Supplementary Table 1), further supporting the role of E2F1 in the G1/S control of the *CCHCR1/TCF19* gene pair. We noticed that *E2F1* knockdown could only reduce *CCHCR1* and *TCF19* expression level by around half (Fig. 2b), it is possible that there are other potential activators regulating the expression of the *CCHCR1/TCF19* gene pair.

Analysis using the "Perturbation" microarray database indicated that *CCHCR1* is co-regulated with *TCF19* in many different experimental perturbations (Supplementary Table 3). In this study, we found that both *CCHCR1* and *TCF19* were up-regulated after CPT treatment (Fig. 6b). CPT is a natural product first isolated from the bark of the Chinese tree, *Camptotheca acuminate*[47]. It was developed as a cancer drug because it specifically inhibits eukaryotic DNA topoisomerase I (topo I) by trapping topo I covalently bound to the DNA. During DNA replication, the replication fork collides with the trapped topo I–DNA complexes, resulting in DNA double-strand breaks, cell arrest, and ultimately apoptosis[15]. CPT causes cell arrest in both S and G2 phase[16]. The arrest pattern depends on the cell cycle distribution of the cells, the concentration and duration of the drug treatment. For instance, cells were arrested mainly in G2 phase when early S phase cells were treated with CPT[48]. On the other hand, exposing cells to low, nanomolar concentration of CPT for a prolonged period (24 h) resulted mainly in S phase accumulation[14]. In our experiment, we found the HeLa cells arrested in both S and G2/M phase after treating with 2 μM CPT for 24 h. However, the majority of the cells were arrested in S phase (67.6% versus 20.8% in the vehicle control) (Fig. 6a). As the expression of the *CCHCR1/TCF19* gene pair is high in S phase, and low in G1 and G2/M phase (Fig. 3c), we suggested that CPT induced the expression of the *CCHCR1/TCF19* gene pair by S phase arrest. Importantly, upon CPT-induced DNA damage, E2F1 is up-regulated, possibly through the activation of the checkpoint kinase 1 (Chk1)[49,50]. Therefore, CPT could also induce the *CCHCR1/TCF19* gene pair through E2F1.

TCF19 codes for a transcription factor that is involved in pancreatic islet proliferation[20]. Depletion of *TCF19* in an insulinoma cell line resulted in a decrease in cell proliferation and an increase in apoptosis[21]. Whether *TCF19* has a role in psoriasis pathogenesis is not known and linkage studies do not suggest its association with psoriasis[51,52]. TCF19 could not regulate *CCHCR1* transcription directly as knockdown of *TCF19* did not affect *CCHCR1* expression (Fig. 5). Although knockdown of *CCHCR1* or *TCF19* lowered the cell count (Fig. 5a), only *CCHCR1* knockdown up-regulated the mRNA expression of cell cycle regulator *p21* (also known as CDKN1A), implying that CCHCR1 and TCF19 may regulate cell growth in divergent pathways. Intriguingly, a recent study indicated that up-regulation of TCF19 can inhibit the expression of *p21* by down-regulating *p21* transcription factor FOXO1[23]. The molecular details of *p21* regulation by the *CCHCR1/TCF19* gene pair is an important question remains to be addressed. p21 is a member of the cyclin-dependent kinase inhibitor family, which regulates cell cycle progression through p16-pRb-E2F-p21 and p53-p21 tumor suppressor pathways[53]. Up-regulation of p21 induces growth arrest[54]. In psoriasis, which displays hyper-proliferation of keratinocytes, expression of p21 protein and mRNA are paradoxically up-regulated[55]. The induction of p21 in psoriasis could be explained by the fact that *CCHCR1* expression in cultured non-lesional psoriatic keratinocytes is down-regulated[5]. Importantly, the bizarre expression of p21 in psoriasis may cause the abnormal differentiation of the keratinocytes. p21 is required for keratinocyte differentiation initiation[56,57]. However, p21 expression is down-regulated at later stages of keratinocyte differentiation because p21 inhibits the expression of terminal differentiation markers[58]. Therefore, persistence high level of p21 may result in abnormal growth and differentiation of the keratinocytes, resulting in psoriasis[10,59]. Further study is required to examine the significance of p21 regulation by CCHCR1 in the pathogenesis of psoriasis.

We and others have recently identified CCHCR1 as a novel component of cytoplasmic P-bodies and centrosome[8,9]. P-bodies travel along the microtubules to and from the centrosome and there is a pair of stationary P-bodies on the centrosome throughout interphase[60,61]. However, how CCHCR1 is involved in the activities of P-bodies and centrosome is still largely unknown. The major interacting partner of CCHCR1 is the mRNA decapping protein EDC4[8]. In the P-bodies, EDC4 regulates mRNA turnover by forming a decapping complex with DCP1, DCP2 and XRN1[62], in which DCP1 and XRN1 are also found to be interacting with CCHCR1[8]. P-bodies numbers and size are dynamic throughout the cell cycle[63]. In S phase, P-bodies number increases and it may be related to the high expression of *CCHCR1* observed in this study. Upon mitotic entry, P-bodies disassemble. It would be interesting to see whether *CCHCR1* expression affects P-bodies dynamics, number, size and activities in the future. The centrosome regulates cell cycle progression and also acts as a microtubule and actin organizing center in the cell[64]. The centrosomal CCHCR1 may involve in cytoskeletal organization[7]. Overexpression of CCHCR1 does not have major effects on the cytoskeletal network. However, after treatment with nocodazole, CCHCR1-overexpressing cells

showed altered actin staining and cell morphology compared with the wildtype[7]. How the S phase-expression of CCHCR1 contributes to the activity of the centrosome is unknown. Duplication of the centrosome happens just before, or at, the onset of S phase[65], coincident with the induction time of CCHCR1 (Fig. 3c). In many cancer cells, loss of centrosome regulation results in centrosome amplification and aneuploidy. E2F1 knockdown suppressed centrosome amplification in $Her2^+$ breast cancer cells, without significantly affecting cell cycle progression. Besides, E2F1 also regulates normal centrosome duplication[66]. It is plausible that E2F1 controls centrosome duplication and amplification through regulating CCHCR1 expression. On the other hand, p21, as a Cdk inhibitor, blocks centrosome duplication, possibly due to the inhibition of cyclin E/cdk2 activity[67,68]. A high level of CCHCR1 in S phase may help to suppress the expression of p21, allowing a favorable environment for centrosome duplication. Taken together, our study suggested that *CCHCR1* is co-regulated with *TCF19* and is induced and highly expressed in the G1/S transition and S phase, possibly by E2F1. Knockdown of *CCHCR1* induced *p21* expression, which may cause the hyper-proliferation and abnormal differentiation of psoriatic keratinocytes in which *CCHCR1* expression is low (Fig. 7). Our study suggested that it is important to understand the role of CCHCR1 in P-bodies and centrosome, especially during S phase, in order to address whether *CCHCR1* is a true disease-causing gene of psoriasis.

Methods

In silico analysis of the intergenic region of *CCHCR1/TCF19* gene pair and genes positively correlated with *CCHCR1*

The intergenic region of *CCHCR1*/*TCF19* was analyzed by Newcpgreport of EMBOSS[69] for the presence of CpG islands and by MatInspector[27] for putative transcription factor binding sites. To search for the genes positively correlated with *CCHCR1* expression, co-expression analysis was done using Genevestigator[28].

Total RNA extraction, first-strand cDNA synthesis, and quantitative PCR

Total RNA from HeLa cells was isolated using TRIzol® Reagent (Life Technologies, USA) according to the manufacturer's instructions. Extracted RNAs were treated with DNase to remove genomic DNA by a TURBO DNA-free™ Kit (Life Technologies, USA), and were reverse transcribed to first-strand cDNAs with random primers using PrimeScript First Strand cDNA Synthesis Kit (Takara, Japan). Quantitative PCR (qPCR) was performed using Fast SYBR Green Master Mix (Life Technologies, USA) in Step One Plus Real-time quantitative PCR system (Life Technologies, USA) and ROX was used as the reference. A final dissociation stage was performed after the thermocycles to verify the specificity of the PCR primers. All amplifications were done in triplicate. The relative expression of gene transcripts was normalized to *RPL13A* expression, and the gene expression levels were calculated using the Pfaffl method[70]. The primer pairs used are listed in Supplementary Table 4.

Overexpression of E2F proteins

To overexpress E2F1, 2 and 3, the coding sequences of the corresponding E2Fs were cloned from cDNA of HeLa cells into pEGFP-N1 vectors. The plasmids were then transfected into HeLa cells using Fugene HD transfection reagent (Roche, Germany) for 48 h to express the corresponding E2F-EGFP fusion proteins. Successful transfection was verified by the GFP expression of the cells.

siRNA transfection

HeLa cells were seeded in a 6-well plate overnight before they were transfected with 2.5 μM of siRNAs using Lipofectamine RNAiMAX transfection reagent (Invitrogen, USA) according to the manufacturer's instructions. *CCHCR1*, *TCF19*, and *E2F1* mRNA were depleted by pre-designed Silencer® Select siRNAs (Ambion, Life Technologies, USA). The negative control siRNA (siCTL) was Silencer® Select Negative Control No. 1 siRNA (#4390843, Ambion, Life Technologies, USA).

Cell cycle synchronization

Cells were synchronized at the G1/S transition by a double thymidine block. In brief, HeLa cells at 25-30% confluency were incubated with 2 mM thymidine in complete DMEM medium for 18 h, released in the thymidine-free complete medium for 9 h and incubated again with 2 mM thymidine in complete DMEM medium for another 17 h. The cells were released from the block by washing with PBS and were then incubated with the complete DMEM medium.

Flow cytometry

To analyze the cell cycle by flow cytometry, cells were trypsinized and fixed in 70% ethanol. Fixed cells were stained with propidium iodide for DNA content and analyzed by flow cytometry using LSRFortessa Cell Analyzer (BD Biosciences, USA).

Dual luciferase assay

DNA fragments containing the *CCHCR1/TCF19* intergenic region with (556 bp) or without (287 bp) exon 1 of both genes were cloned from HeLa cell genomic DNA and inserted into the firefly luciferase (Fluc) reporter vector pGL4.17 (Promega, USA), in either the forward or reverse condition, to examine the activity of the *CCHCR1* and *TCF19* promoters, respectively.

Firefly luciferase activity was measured to test the transcription activities of different *CCHCR1/TCF19* intergenic DNA fragments at different phases of the cell cycle. In brief, HeLa cells ($1x10^6$) were seeded in 24-well plates and incubated for 24 h before transfection. Double transfections were performed using 200 ng total DNA containing 100 ng of Fluc reporter vector and 100 ng of Renilla luciferase vector (pRL-TK; as an internal control; Promega). After 4 h of transfection, cells were synchronized by double thymidine treatment and harvested at different cell cycle points: 0 h (G1/S), 2 h (S), 8 h (G2/M) and 14 h (G1) after release from the thymidine block. Cells were lysed and luciferase activity was determined using a Dual-Luciferase Reporter Assay System (Promega) with a GloMax-96 Microplate Luminometer (Promega) as previously described[71]. The Fluc activity was normalized to the Renilla luciferase (Rluc) (Fluc/Rluc ratio) in each individual experiment to correct for differences in transfection efficiency. In addition, the Fluc/Rluc ratio of each of the promoter fragments was subtracted by the Fluc/Rluc ratio of the empty vector control (pGL4.17).

Cell cultures, trypan blue exclusion assay and camptothecin (CPT) treatment

Human cervical cancer HeLa cells (ATCC® CRM-CCL-2™) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and maintained at 37°C in a humidified atmosphere of 5% CO_2. Cell growth was measured by trypan blue exclusion assay[72]. For camptothecin (CPT) treatment, HeLa cells were seeded in a 6-well plate overnight and treated with 0.1% DMSO (vehicle control) or 2 μM CPT for 24 hours. Cells were then collected for flow cytometry and qPCR analysis.

Statistical analysis

Statistical analysis was performed with GraphPad Prism software version 7.0 (GraphPad Software, USA). Data are presented as mean value and standard deviation. Paired t-test was used to determine the significance of the data comparing with the control. Statistical significance was defined as $P < 0.05$.

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Acknowledgements

The authors would like to thank Prof. Z. X. Lin of the School of Chinese Medicine for technical assistance. We would also like to express our gratitude for the special allowance and funding provided by the School of Life Sciences and School of Biomedical Sciences to continue this research.

Author contributions statement

YHL, YYC, and WKL conceived the experiments; YHL, YYC, and WKL conducted the experiments; YHL, YYC and WKL analyzed the results; YHL, KMC, KNL and WKL reviewed the manuscript.

Data availability statement

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Additional information

We declare NO conflicts of interest.

Figure Legend

Figure 1. Genome arrangement of the *CCHCR1* gene. (**a**) Cen.: Centromeric side, Tel.: Telomeric side. (**b**). Putative E2F binding sites (grey arrowhead) and CpG island in the *CCHCR1*/*TCF19* locus (-1231 to +638. Numbers refer to the position related to the transcription start site (+1) of *CCHCR1* (NCBI Reference Sequence: NM_019052.3).

Figure 2. E2F1 induced the expression of *CCHCR1* and *TCF19*. (**a**) RT-qPCR for the expression of *CCHCR1* and *TCF19* in HeLa cells transfected with E2F activators (E2F1, E2F2 or E2F3). pEGFP-N1 was the empty vector control. Gene expression level was normalized to the expression level of *RPL13A*. Results were mean ± SD of three replicates. Statistically significant differences of the gene expression levels were compared to empty vector control and were analyzed by paired t-test (** = P ≤ 0.01). (**b**) Down-regulation of *CCHCR1* and *TCF19* mRNA by E2F1 knockdown. Cells were transfected with siE2F1 and were harvested for the time indicated (time post-transfection). mRNA expressions (normalized to *RPL13A* gene expression) were analyzed by RT-qPCR. Results were mean ± SD of three replicates. Statistically significant differences of the gene expression levels were compared to 0 h post-siE2F1 transfection and were analyzed by paired t-test (*** = P ≤ 0.001).

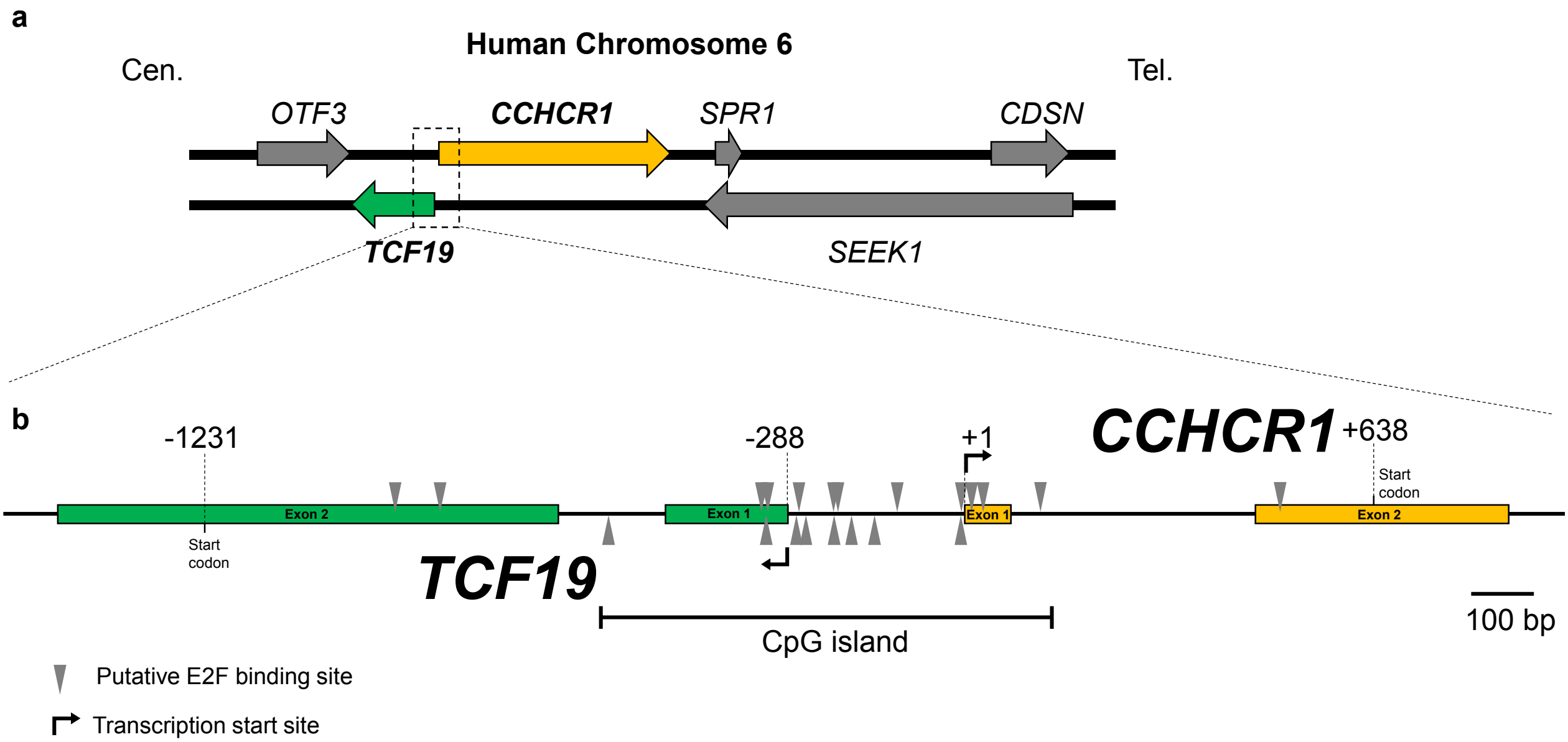
Figure 3. *CCHCR1* and *TCF19* are up-regulated in G1/S transition. (**a**) HeLa cells were synchronized and arrested in G1/S transition by a double thymidine block. The cells were released and collected at different time points, stained with propidium iodide (PI). The percentage of cells in different cell cycle phases in each time point (G1/S, S, G2/M, and G1) were analyzed by flow cytometry. (**b**) Percentage of cells in different cell cycles after double thymidine block and release. (C) Relative expression of *CCHCR1* and *TCF19* in different cell cycle phases. *CCHCR1* and *TCF19* gene expression were normalized to *RPL13A* gene expression. The time points after release from the double thymidine block were indicated for different cell cycle phases; i.e. 0 h: G1/S, 2 h: S, 8 h: G2/M and 14 h: G1. Results were mean ± SD of three replicates. Statistically significant differences of the gene expressions were compared to 0 h after double thymidine release (G1/S transition) and were analyzed by paired t-test (** = P ≤ 0.01 and *** = P ≤ 0.001)

Figure 4. Activity of *CCHCR1* and *TCF19* promoters in different cycle cell phases. (**a**) Promoter fragments of *CCHCR1* and *TCF19* were cloned into pGL4.17 vector to examine their activities by luciferase assay. (**b**) The ratio of Firefly to Renilla luciferase activity (Fluc/Rluc) for each promoter fragments was subtracted with the Fluc/Rluc ratio of the empty vector control (pGL4.17). Results are mean ± SD of three replicates. Statistically significant differences of the promoter activities were compared to 14 h after double thymidine release (G1 phase) and were analyzed by paired t-test (* = ≤0.05, ** = P ≤ 0.0 1 and *** = P ≤ 0.001).

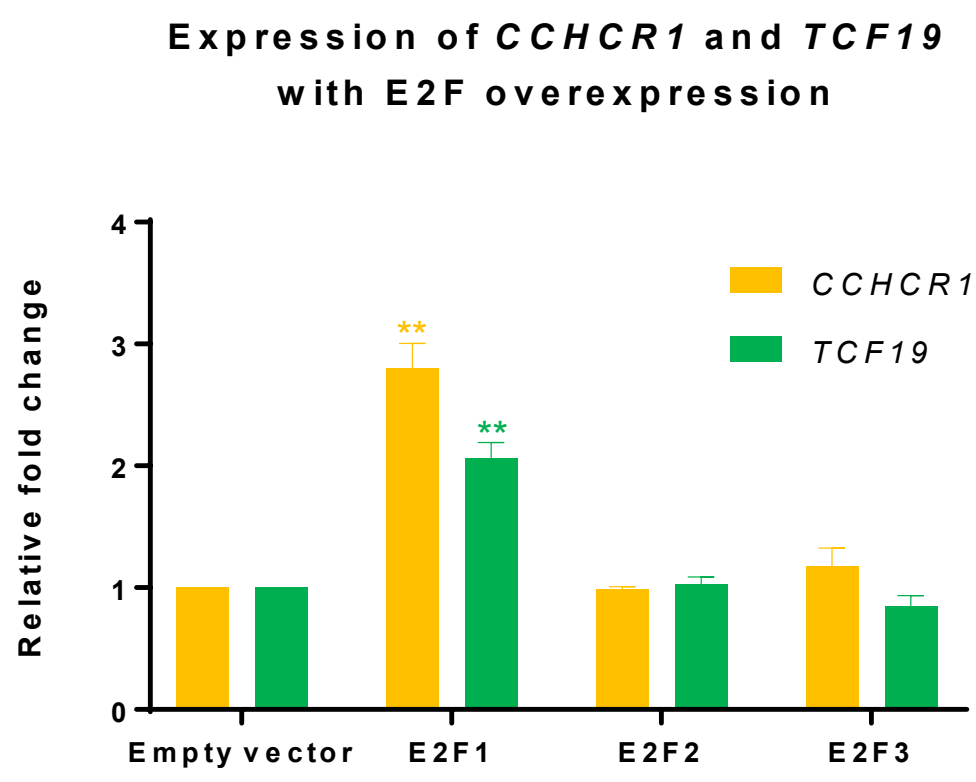
Figure 5. *CCHCR1* or *TCF19* knockdown affected the growth of HeLa cells. (**a**) HeLa cells were transfected with the corresponding siRNAs. Non-viable cells were counted using trypan blue exclusion assay. siCTL was Silencer select negative control #1 (Ambion, Life Technologies, USA). Results are mean ± SD of five replicates. Statistically significant differences of the cell counts were compared to the untransfected samples in the corresponding time point, and were analyzed by paired t-test (* = ≤0.05 and ** = P ≤ 0.01). (**b**) Up-regulation of *p21* mRNA was observed after knockdown of *CCHCR1*. siRNAs were transfected into HeLa cells and were collected for analysis 4 days post-transfection. Gene expressions (normalized to *RPL13A* gene expression) were analyzed by RT-qPCR. Results are mean ± SD of three replicates. Statistically significant differences of the gene expressions were compared to untransfected sample and were analyzed by paired t-test (* = ≤0.05, ** = P ≤ 0.01 and *** = P ≤ 0.001).

Figure 6. Camptothecin (CPT) up-regulated *CCHCR1* and *TCF19* by inducing S phase arrest. (**a**) HeLa cells were treated with 0.1% DMSO (vehicle control) or 2 μM CPT for 24 hours. The percentage of cells in different cell cycle phases were analyzed by flow cytometry. (**b**) Relative expression of *CCHCR1* and *TCF19* (normalized to *RPL13A* gene expression) in HeLa cells after the treatment with CPT for 24 hours. Results are mean ± SD of three replicates. Statistically significant differences of the gene expression level were compared to 0.1% DMSO treatment and were analyzed by paired t-test (* = ≤0.05 and ** = P ≤ 0.01).

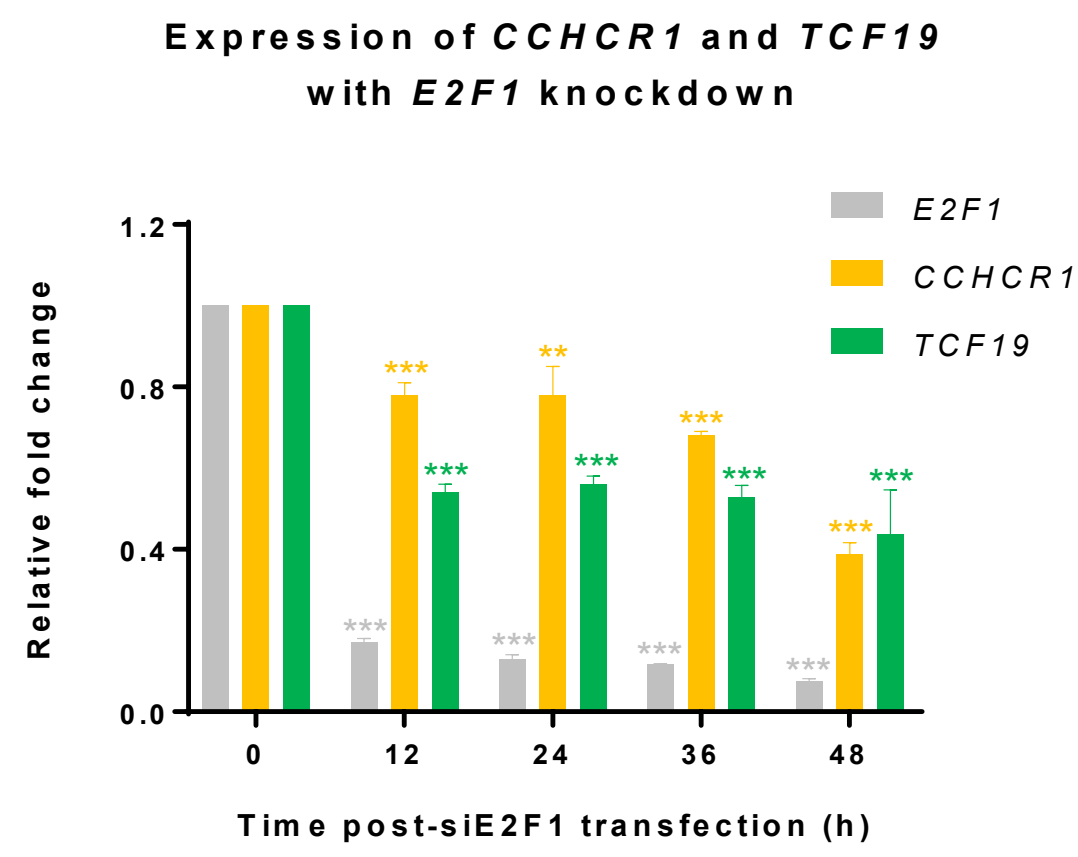
Figure 7. Proposed pathway of E2F1-mediated induction of the *CCHCR1/TCF19* gene pair. In G1/S transition, E2F1 may induce the expression of *CCHCR1* and *TCF19* by binding to the *CCHCR1/TCF19* bidirectional promoter. Both CCHCR1 and TCF19 is important for cell growth. CCHCR1 may control the increase of P-bodies and centrosome duplication in S phase. CCHCR1 inhibits the expression of *p21*. p21 causes growth arrest. However, in psoriasis, high p21 expression may cause hyper-proliferation and abnormal differentiation of the keratinocytes. Besides, p21 inhibits centrosome duplication.

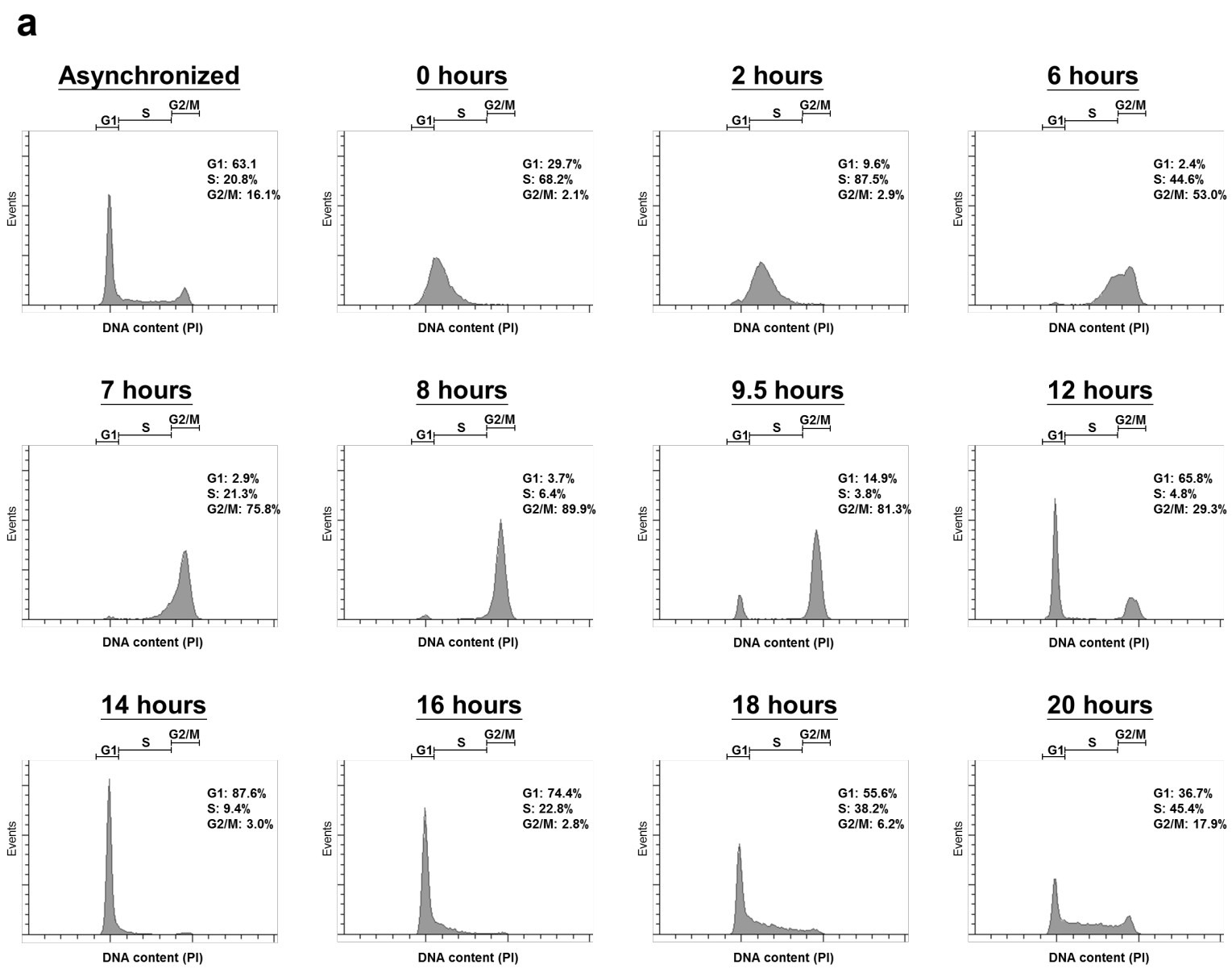

a
Human Chromosome 6
Cen.
Tel.
OTF3
CCHCR1
SPR1
CDSN
TCF19
SEEK1
b
-1231
-288
+1
CCHCR1
+638
Start codon
Exon 2
Exon 1
Exon 1
Exon 2
Start codon
TCF19
CpG island
100 bp
Putative E2F binding site
Transcription start site

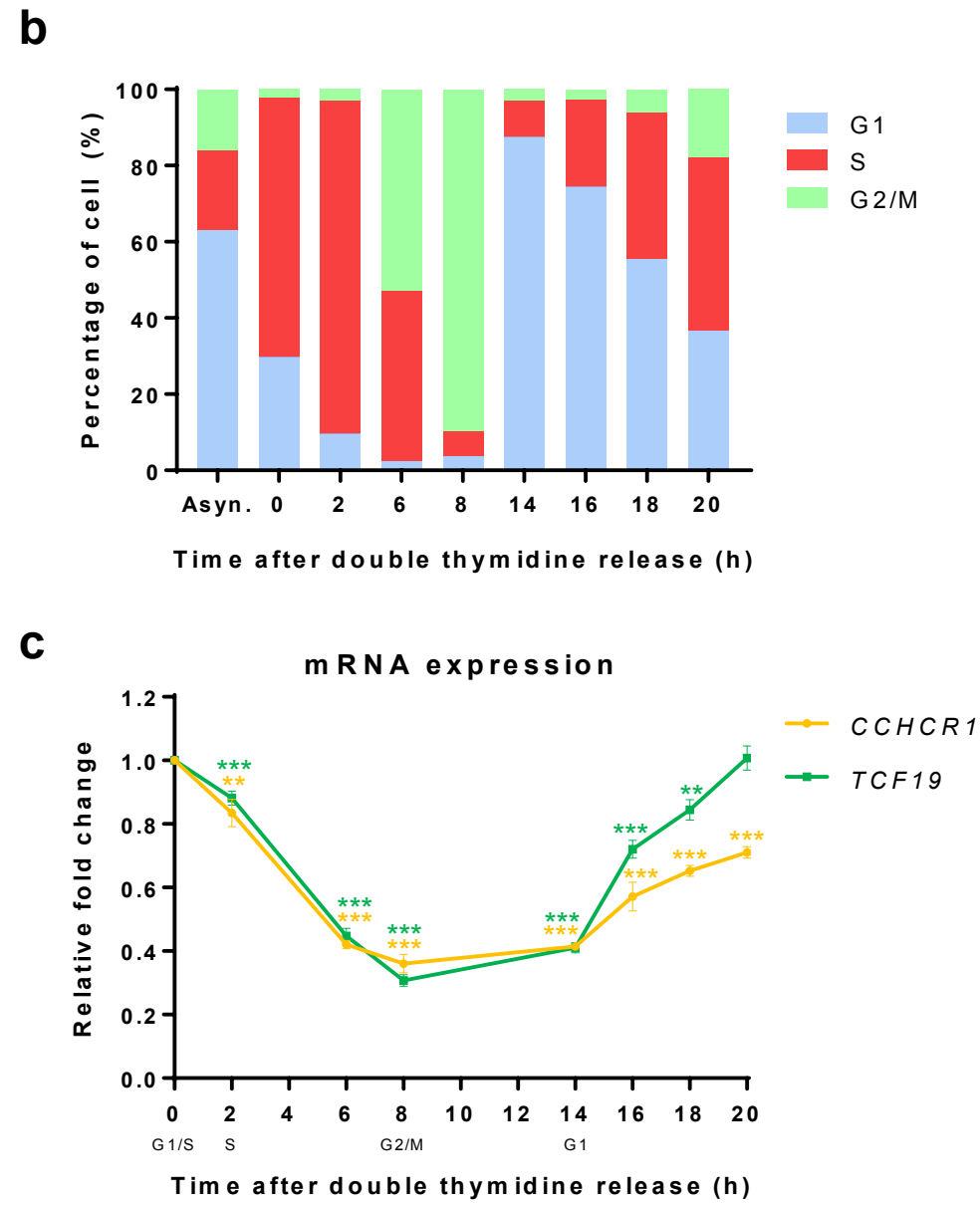
a

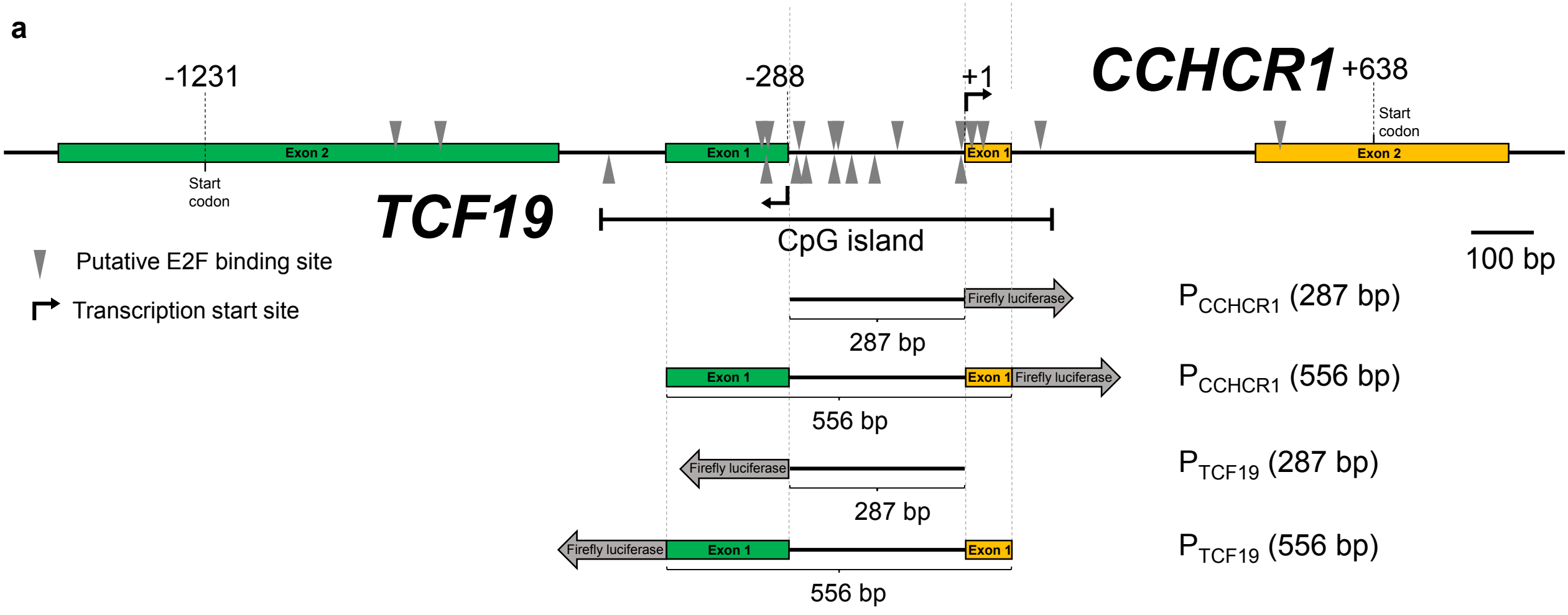

Expression of CCHCR1 and TCF19
with E2F overexpression
Relative fold change
4
3
2
1
0
CCHCR1
TCF19
**
**
Empty vector
E2F1
E2F2
E2F3

b


Expression of CCHCR1 and TCF19
with E2F1 knockdown
Relative fold change
1.2
0.8
0.4
0.0
E2F1
CCHCR1
TCF19
***
0
12
24
36
48
Time post-siE2F1 transfection (h)


a
Asynchronized
G1: 63.1
S: 20.8%
G2/M: 16.1%
0 hours
G1: 29.7%
S: 68.2%
G2/M: 2.1%
2 hours
G1: 9.6%
S: 87.5%
G2/M: 2.9%
6 hours
G1: 2.4%
S: 44.6%
G2/M: 53.0%
7 hours
G1: 2.9%
S: 21.3%
G2/M: 75.8%
8 hours
G1: 3.7%
S: 6.4%
G2/M: 89.9%
9.5 hours
G1: 14.9%
S: 3.8%
G2/M: 81.3%
12 hours
G1: 65.8%
S: 4.8%
G2/M: 29.3%
14 hours
G1: 87.6%
S: 9.4%
G2/M: 3.0%
16 hours
G1: 74.4%
S: 22.8%
G2/M: 2.8%
18 hours
G1: 55.6%
S: 38.2%
G2/M: 6.2%
20 hours
G1: 36.7%
S: 45.4%
G2/M: 17.9%
G1
S
G2/M
Events
DNA content (PI)


b
Percentage of cell (%)
100
80
60
40
20
0
Asyn.
0
2
6
8
14
16
18
20
Time after double thymidine release (h)
G1
S
G2/M
c
mRNA expression
Relative fold change
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
14
16
18
20
G1/S
S
G2/M
G1
Time after double thymidine release (h)
CCHCR1
TCF19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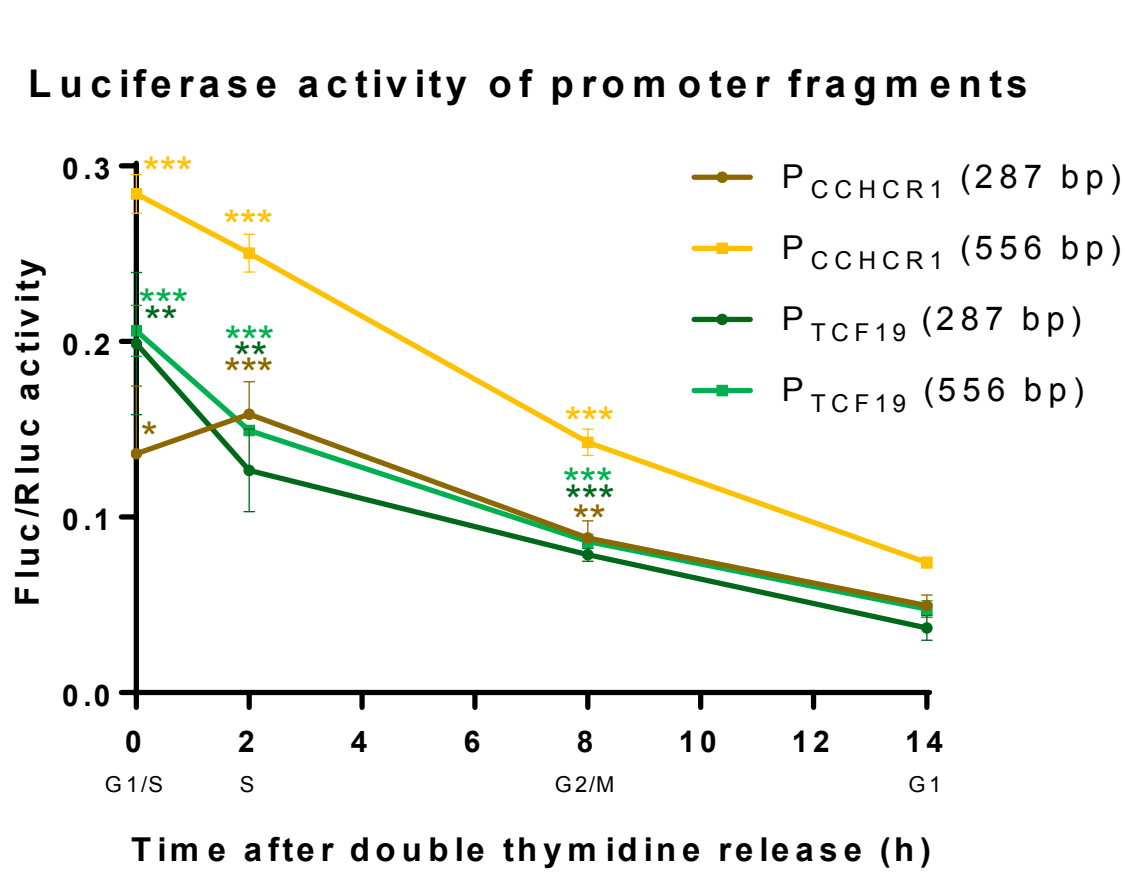
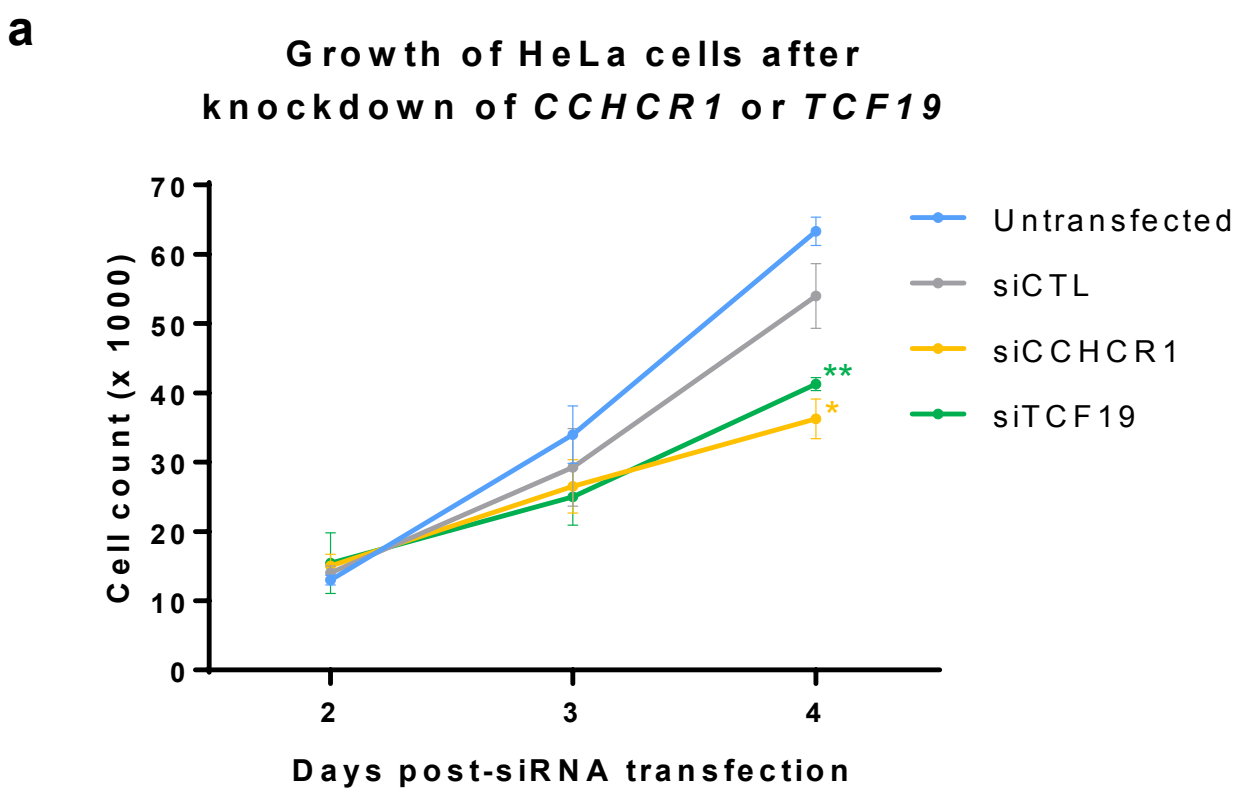
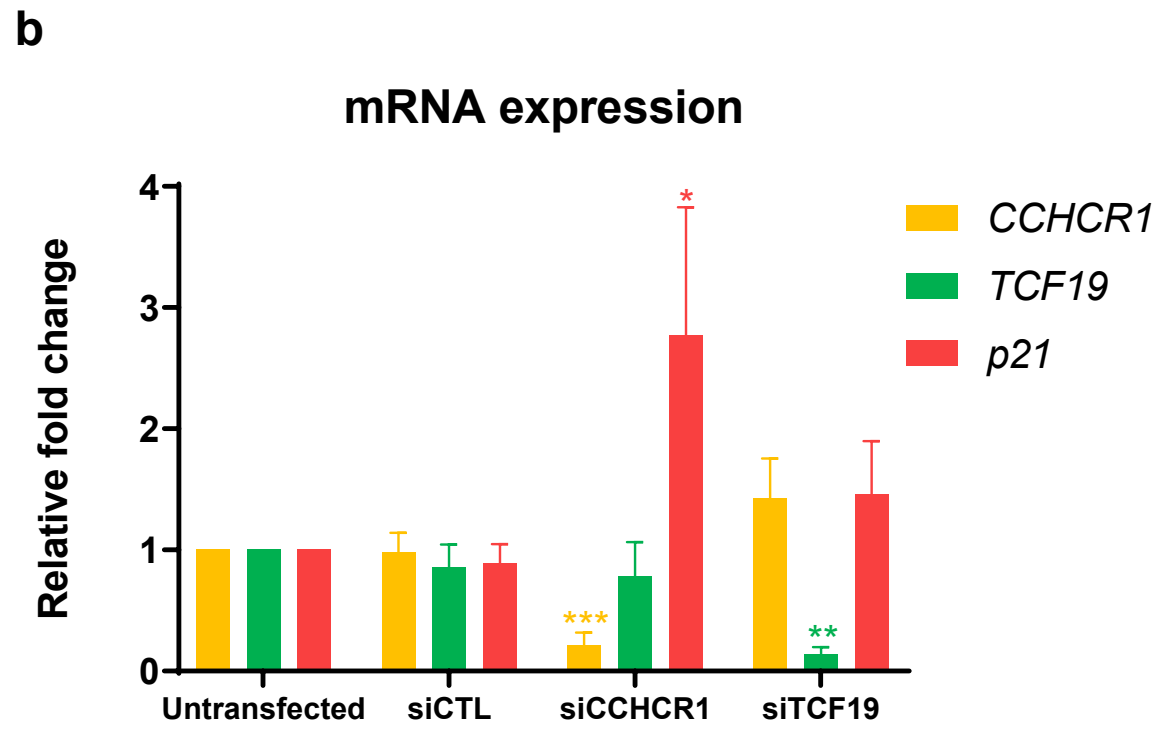
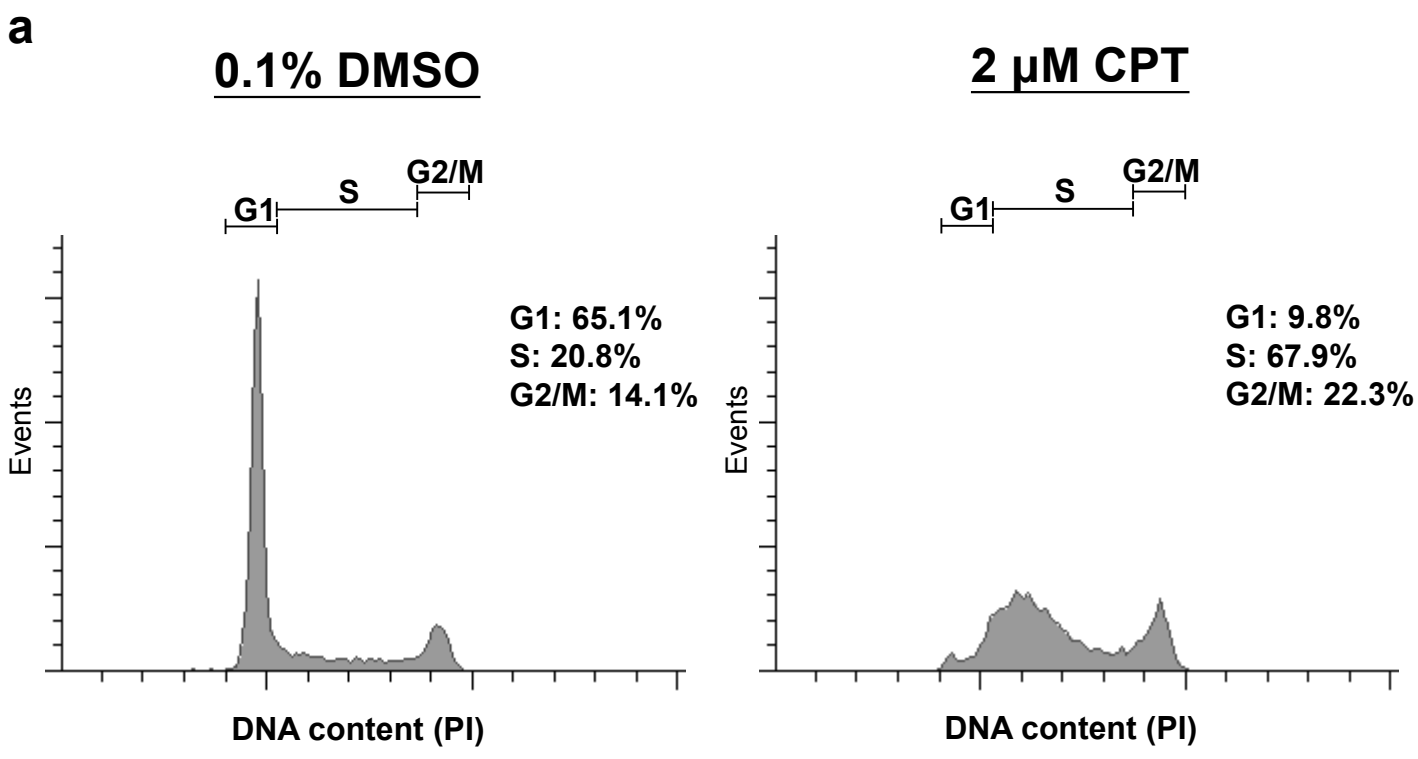
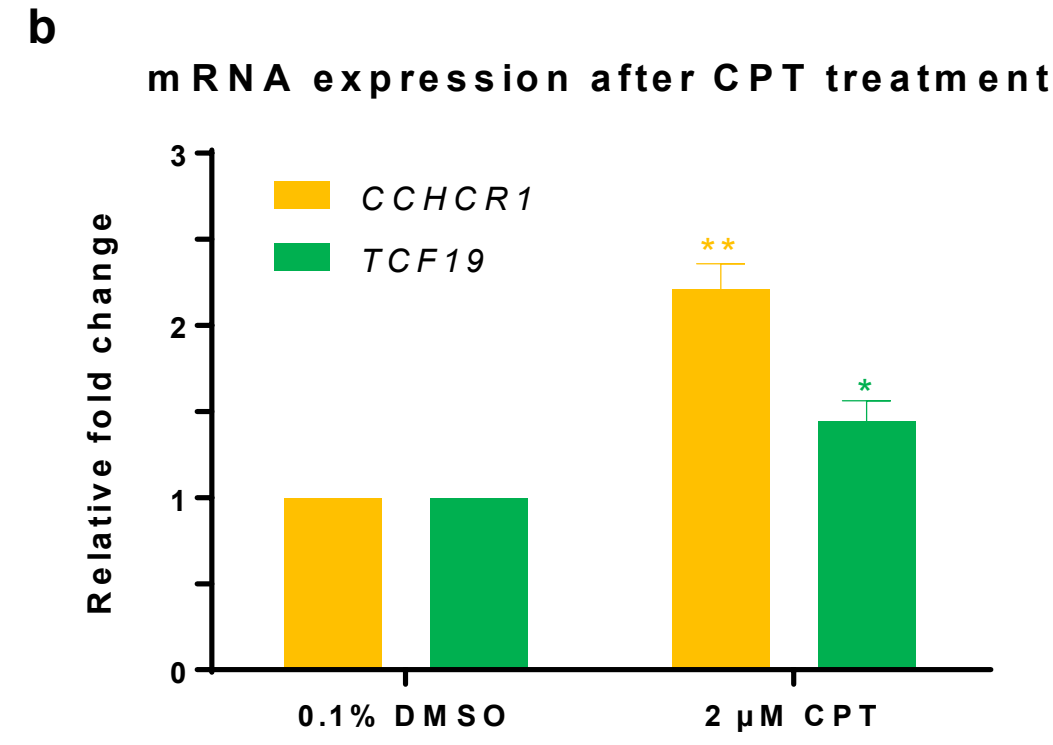
-1231
-288
+1
CCHCR1
+638
Start codon
Exon 2
Exon 1
Exon 1
Exon 2
Start codon
TCF19
CpG island
100 bp
Putative E2F binding site
Transcription start site
Firefly luciferase
287 bp
P CCHCR1 (287 bp)
Exon 1
Exon 1
Firefly luciferase
556 bp
P CCHCR1 (556 bp)
Firefly luciferase
287 bp
P TCF19 (287 bp)
Firefly luciferase
Exon 1
Exon 1
556 bp
P TCF19 (556 bp)

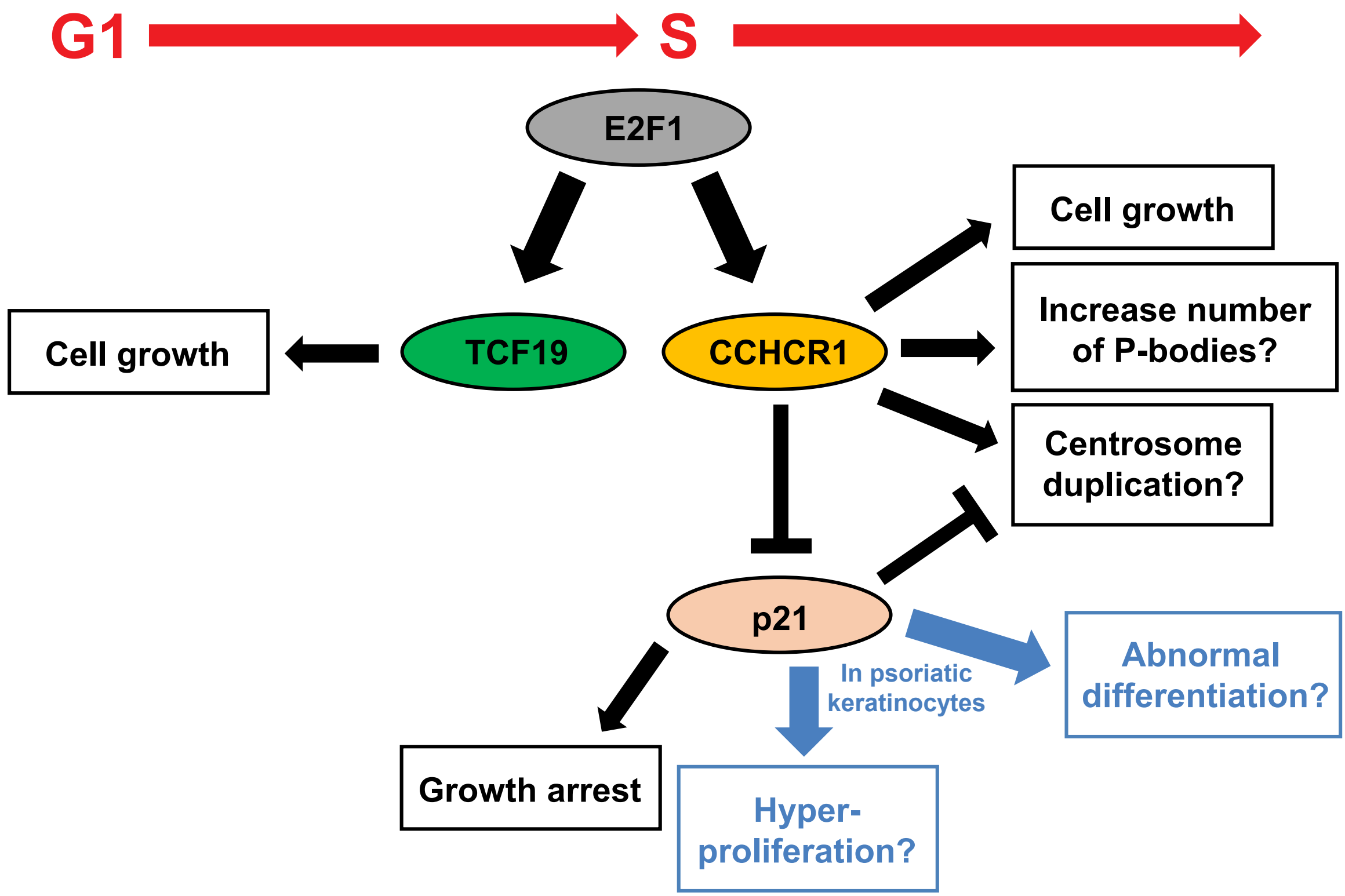

b
Luciferase activity of promoter fragments
Fluc/Rluc activity
0.3
0.2
0.1
0.0
0
2
4
6
8
10
12
14
G1/S
S
G2/M
G1
Time after double thymidine release (h)
P CCHCR1 (287 bp)
P CCHCR1 (556 bp)
P TCF19 (287 bp)
P TCF19 (556 bp)


a
Growth of HeLa cells after knockdown of CCHCR1 or TCF19
Cell count (x 1000)
70
60
50
40
30
20
10
0
2
3
4
Days post-siRNA transfection
Untransfected
siCTL
siCCHCR1
siTCF19
**
*


b
mRNA expression
Relative fold change
4
3
2
1
0
Untransfected
siCTL
siCCHCR1
siTCF19
CCHCR1
TCF19
p21
***
**
*


a
0.1% DMSO
2 μM CPT
G1
S
G2/M
G1: 65.1%
S: 20.8%
G2/M: 14.1%
G1: 9.8%
S: 67.9%
G2/M: 22.3%
Events
DNA content (PI)


b
mRNA expression after CPT treatment
CCHCR1
TCF19
Relative fold change
0
1
2
3
**
*
0.1% DMSO
2 μM CPT


G1
S
E2F1
Cell growth
TCF19
CCHCR1
Cell growth
Increase number of P-bodies?
Centrosome duplication?
p21
Growth arrest
In psoriatic keratinocytes
Hyper-proliferation?
Abnormal differentiation?