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RNF20 inhibits TFIIS-facilitated transcriptional elongation to suppress pro-oncogenic gene expression

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Summary

hBRE1/RNF20 is the major E3 ubiquitin ligase for histone H2B. RNF20 depletion causes a global reduction of monoubiquitylated H2B (H2Bub) levels and augments the expression of growth promoting, pro-oncogenic genes. Those genes reside preferentially in compact chromatin, and are inefficiently transcribed under basal conditions. We now report that RNF20, presumably via H2Bub, represses selectively those genes by interfering with chromatin recruitment of TFIIS, a factor capable of relieving stalled RNA polymerase II. RNF20 inhibits the interaction between TFIIS and the PAF1 complex and hinders transcriptional elongation. TFIIS ablation selectively abolishes the upregulation of those genes upon RNF20 depletion, and attenuates the cellular response to EGF. Consistent with its positive role in transcription of pro-oncogenic genes, TFIIS expression is elevated in various human tumors. Our findings provide a molecular mechanism for selective gene repression by RNF20, and position TFIIS as a key target of RNF20's tumor suppressor activity.

Introduction

The eukaryotic DNA is packaged into a chromatin structure consisting of repeating nucleosomes, comprising 146 base pairs of DNA wrapped around an octamer of core histone proteins. The histone tails are subjected to several post-translational modifications (PTM), which are crucial in regulation of transcription, DNA repair and other chromatin-associated processes (Berger, 2007; Jenuwein and Allis, 2001; Weake and Workman, 2008). One such PTM is the monoubiquitylation of histone H2B on lysine 123 in yeast, or lysine 120 in mammals, carried out by the yeast E3 ligase BRE1, or the mammalian RNF20(hBRE1)/RNF40 complex (Hwang et al., 2003; Kim et al., 2005; Zhu et al., 2005). The ubiquitin-conjugating (E2) enzymes involved in that process were reported to be RAD6 in yeast, and its mammalian orthologs RAD6A and RAD6B (Kim et al., 2009; Robzyk et al., 2000).

Transcription by RNA polymerase II is a highly regulated process. This applies equally not only to the extensively studied transcription initiation step, but also to the elongation step. During elongation, RNA polymerase II (Pol II) may often become paused or arrested, in a

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manner that can be rate limiting for efficient transcription (Rahl et al., 2010; Selth et al., 2010; Sigurdsson et al., 2010). Many elongation factors are required to stimulate Pol II's progression; some are general and apply to many genes, while others are selective to specific subsets of genes (Arndt and Kane, 2003; Reines et al., 1999; Selth et al., 2010).

TFIIS is a transcription elongation factor that is highly conserved in the eukaryotic and archaeal kingdoms. TFIIS helps Pol II pass through transcriptional blocks on template DNA. It can reactivate arrested elongation complexes by stimulating endonucleolytic cleavage by Pol II of the nascent RNA (Fish and Kane, 2002). This activity of TFIIS is required for efficient read-through at arrested sites (Ghavi-Helm et al., 2008; Labhart and Morgan, 1998; Reinberg and Roeder, 1987). It is also suggested that TFIIS has a role in overcoming the arrest induced by nucleosomes, thereby promoting chromatin transcription *in vitro* (Guermah et al., 2006; Kireeva et al., 2005). Genome-wide analysis revealed that TFIIS plays a role also in RNA polymerase III transcription (Ghavi-Helm et al., 2008).

In the yeast *S.cerevisiae*, TFIIS is encoded by *DST1*, and is not essential for viability under normal conditions. *DST1* deletion mutant strains grow like wild type, but are sensitive to drugs that compromise elongation efficiency (Nakanishi et al., 1992; Nakanishi et al., 1995; Ubukata et al., 2003). Recently, it was shown that a yeast TFIIS mutant that lacks transcript cleavage stimulatory activity and inhibits Pol II unstimulated cleavage is lethal, indicating that escape from transcriptional arrest via transcript cleavage is essential for efficient transcript elongation and cell viability (Sigurdsson et al., 2010). In mammals, two different forms of TFIIS were originally identified: a ubiquitously expressed form encoded by the *TCEA1* gene, and a testis-specific form encoded by *TCEA2* (Xu et al., 1994). A third form, encoded by *TCEA3*, was later identified in vertebrates (Labhart and Morgan, 1998). Knockout of mouse *TCEA1* leads to embryonic lethality (Ito et al., 2006).

Several studies in yeast and mammals addressed a role for RNF20 and H2B ubiquitylation in regulation of transcription in general, and of transcriptional elongation in particular. In yeast, BRE1, RAD6 and consequently monoubiquitylated H2B (H2Bub) were shown to associate with elongating Pol II (Xiao et al., 2005). H2Bub was found to be necessary for efficient reassembly of nucleosomes and restoration of the chromatin structure during the elongation process (Osley et al., 2006). In mammals, H2Bub was found to associate preferentially with the transcribed region of highly expressed genes (Minsky et al., 2008), and *in vitro* transcription elongation assays established a role for H2Bub in facilitating elongation by Pol II (Pavri et al., 2006). Moreover, H2B ubiquitylation was recently shown to disrupt chromatin compaction in an *in vitro* system of chemically defined nucleosome arrays (Fierz et al., 2011). Other studies, however, have suggested a negative role for H2Bub in regulating transcriptional elongation. For example, yeast H2Bub was shown to serve as a barrier to transcriptional elongation by blocking the recruitment of the kinase Ctk1, which phosphorylates Pol II on serine 2 of its carboxy terminal domain (CTD) repeat (Wyce A, 2007). Recent work implicated H2Bub in enhancing nucleosome stability, proposing that this is a mechanism by which ubH2B plays a role in gene repression (Chandrasekharan et al., 2009). Studies in mammals showed that RNF20 suppresses the induction of interferon regulatory factor 1 (IRF1) in response to STAT1 signaling (Buro et al., 2010). Furthermore, H2Bub was implicated in gene repression through USP22, an H2Bub-specific deubiquitylating enzyme and a subunit of the human SAGA complex, which is necessary for activation of SAGA-dependent genes and contributes to heterochromatin silencing (Zhang et al., 2008; Zhao et al., 2008).

Previously, we showed that RNF20 selectively regulates a subset of genes in human cells and acts as a putative tumor suppressor. Genes whose expression was positively regulated by RNF20 (RNF20-dependent genes) include the *p53* tumor suppressor as well as numerous

histone H2A and H2B genes. In contrast, RNF20 suppresses the expression of several proto-oncogenes and growth-related genes, including many genes that are induced by epidermal growth factor (EGF). RNF20-suppressed genes are characterized by a rather low rate of transcription, despite high occupancy by Pol II. Notably, these genes are preferentially associated with compact chromatin fibers, which presumably makes them more difficult to transcribe and may result in frequent pausing of Pol II at multiple points along the transcribed gene (Shema et al., 2008). Given the reported role of TFIIS in relieving Pol II pausing and facilitating transcriptional elongation through “difficult” regions, we asked whether it might be involved in the positive regulation of RNF20-suppressed genes. We now describe a novel mechanism by which RNF20, presumably via H2B ubiquitylation, represses transcription elongation through inhibition of TFIIS recruitment to chromatin. Depletion of TFIIS abolishes RNF20-mediated suppression of growth-related genes, and attenuates the cellular response to EGF. Our findings thus position TFIIS as a key target in RNF20-mediated gene repression, and propose a molecular mechanism that mediates the tumor suppressor activity of RNF20.

Results

Transcriptional regulation by RNF20 requires its E3 ligase activity

We have previously shown that RNF20 selectively regulates the expression of a subset of genes in human cells (Shema et al., 2008). These genes can be divided into two distinct subgroups: (1) genes that are dependent on RNF20 for their optimal expression, and (2) genes that are suppressed by RNF20. Genes representative of each subgroup are shown in Figure 1A: The RNF20-dependent genes *H2BD*, *HOXA10* and *TP53* (*p53*) are strongly downregulated upon depletion of RNF20 by siRNA, while the RNF20-suppressed genes *NR4A2*, *C-FOS*, *FOSL2*, *C-MYC* and others are upregulated. As shown earlier, this is due to altered transcription rates (Shema et al, 2008). Of note, the bulk transcriptome of the cells, represented here by *GAPDH* and *NDUFA6*, is not significantly affected by transient RNF20 depletion, suggesting that H2B ubiquitylation is not rate limiting for global transcription (Shema et al, 2008). These effects were validated with 3 different RNF20 siRNA oligonucleotides (Supplemental Fig. S1A,B), ruling out off-target effects.

Next, we sought to determine whether transcriptional regulation of those genes by RNF20 requires its E3 ubiquitin ligase activity. To that end, we knocked down endogenous RNF20 using siRNA directed against its 5' UTR and reintroduced exogenous wild type (wt) RNF20 or a mutant RNF20 lacking the RING domain (Δ RING; dR). Knockdown of endogenous RNF20 in combination with an empty vector led to a marked decrease in H2Bub levels (Figure 1B), which was largely rescued by reintroduction of wt RNF20 but not the catalytically inactive Δ RING (dR) mutant. Reintroduction of wt RNF20 reversed the transcriptional effects of RNF20 depletion on both gene subgroups (Fig. 1C). However, this did not occur with the Δ RING mutant, suggesting that RNF20 regulates gene expression in a manner dependent on its E3 ligase activity, possibly through monoubiquitylation of histone H2B.

RAD6A is the main E2 enzyme recruited by RNF20, and is responsible for RNF20-mediated H2B ubiquitylation (Kim et al., 2009). Indeed, RAD6A knockdown led to a strong reduction in H2Bub levels, comparable to that seen upon RNF20 knockdown (Fig. 1D; knockdown validation in Fig. 1E). Notably, RAD6A depletion reproduced faithfully the transcriptional effects of RNF20 knockdown (Fig. 1E), further supporting the conclusion that RNF20-mediated transcriptional regulation depends on its ubiquitin ligase activity. Moreover, knockdown of RNF40, the heterodimeric partner of RNF20 that together with it forms the active E3 ligase, effectively inhibited H2B monoubiquitylation and also reproduced the selective effects of RNF20 knockdown on gene expression (Supplemental

Fig. S1C,D). However, the interpretation of this result is limited by the fact that RNF40 depletion also caused a marked reduction in RNF20 protein (Supplemental Fig. S1C), suggesting that formation of an RNF20-RNF40 dimer is necessary for stabilization of each partner in the dimer, in line with earlier observations (Kim et al., 2009).

RNF20 and TFIIS counter the transcriptional effects of each other on the expression of a specific subset of genes

RNF20-suppressed genes reside preferentially in closed chromatin and are poorly transcribed despite bearing histone marks usually associated with active transcription and despite their high association with RNA-polymerase II, supporting the possibility that their transcription is being persistently hindered (Shema et al., 2008). Given the known function of TFIIS in relieving transcriptional blocks, we wished to determine whether TFIIS can selectively modulate the transcription of this subgroup of genes. In addition to *TCEA1*, the most prominent TFIIS member in HeLa cells, these cells express also moderate amounts of *TCEA2* mRNA, but negligible amounts of *TCEA3* mRNA (data not shown). We therefore performed a combined knockdown of RNF20 and TFIIS (*TCEA1* plus *TCEA2* genes) followed by qRT-PCR analysis of mRNA levels. Knockdown validation is shown in Figure 2A and Supplemental Fig. S2A. Of note, whereas RNF20 depletion exerted the expected effects on the RNF20-suppressed, RNF20-independent and RNF20-dependent genes (Fig 2, panels B,C and D, respectively; compare siLacZ to siRNF20), TFIIS depletion by itself did not affect the basal expression levels of any of those genes (compare siLacZ to siTCEA1+2), implying that basal transcription in this experimental system is not dependent on TFIIS. However, TFIIS depletion abrogated almost completely the positive effect of RNF20 knockdown on the RNF20-suppressed genes (Fig. 2B, compare siRNF20 to siTCEA1+2+siRNF20), while making no difference to the other subgroups (Fig. 2C,D). Analysis of heterogeneous nuclear RNA with intron-derived primers, which provides a better representation of relative transcription rates (Kuroda et al., 2005; Phelps et al., 2006), revealed an essentially similar picture (Supplemental Fig. S2B), strongly arguing that TFIIS is required for efficient transcription of the RNF20-repressed genes upon RNF20 depletion. Knockdown of either *TCEA1* or *TCEA2* alone had a partial effect (Supplemental Fig. S2C,D), indicating that both contribute to the transcriptional response in these cells, as opposed to knockdown of *TCEA3* which had no effect (Supplemental Fig. S2E,F). Hence, TFIIS is selectively required for the transcriptional induction of the RNF20-suppressed genes following RNF20 knockdown.

The involvement of TFIIS in the regulation of RNF20-suppressed genes was further assessed by overexpression experiments. Augmentation of TFIIS levels by overexpression of either HA-tagged or flag-tagged *TCEA1* did not affect RNF20 or H2Bub levels (Fig. 3A). Notably, excess TFIIS elicited a modest upregulation of all tested RNF20-suppressed mRNAs (Figure 3B), while not affecting RNF20-independent genes (*NDUFA6*) or RNF20-dependent genes (*p53* and *H2BD*; Figure 3C). Thus, RNF20 and TFIIS selectively exert opposing effects on the transcriptional regulation of RNF20-suppressed genes.

RNF20 depletion increases TFIIS occupancy on chromatin

To further elucidate the crosstalk between TFIIS and RNF20 in the transcriptional regulation of RNF20-suppressed genes, we examined the effect of RNF20 knockdown on TFIIS. We found that while RNF20 depletion had no appreciable effect on overall TFIIS levels (Figure 4A, compare lanes 1 and 2; see also Supplemental Fig. S2A), it increased strongly the association of TFIIS with chromatin (Figure 4B, compare lanes 3 and 4).

Consistent with the increased global association of TFIIS with chromatin in RNF20-depleted cells, chromatin immunoprecipitation (ChIP) analysis confirmed the enhanced recruitment

of TFIIS to the transcribed regions of numerous genes following RNF20 knockdown (Figure 4C). This increase was global and was observed not only for the RNF20-suppressed genes but actually for all genes tested. This implies that RNF20, presumably through H2B ubiquitylation, can interfere with the recruitment of TFIIS to chromatin. While this interference is general, only the RNF20-suppressed genes appear to rely on TFIIS for their efficient transcription (Fig. 2 C-E); hence, they selectively benefit from the relief of this interference and the resultant increased recruitment of TFIIS. In sum, TFIIS likely provides a distinct transcription-facilitating function that is essential for the RNF20-suppressed genes but is dispensable for the other subgroups of genes.

Downregulation of RNF20 enables more efficient elongation of RNF20-suppressed genes and increases the interaction between TFIIS and the PAF1 complex

Since TFIIS is an elongation factor, we next sought to examine the rate of elongation of RNF20-suppressed genes following RNF20 depletion alone or in combination with TFIIS depletion. Knockdown of RNF20 alone augmented the rate of transcriptional elongation of RNF20-suppressed genes, as deduced from the increase in the ratio between RNA polymerase II occupancy near the 3' end relative to its occupancy near the 5' end of a gene (Figure 5A). This suggests a negative role for RNF20/H2Bub in regulation of transcriptional elongation. This effect was not observed for RNF20-independent genes such as *GAPDH* and *NDUFA6* implying that it is specific for RNF20-repressed genes. Importantly, in line with the transcriptional effects shown in Figure 2, the increase in elongation rate was abolished when RNF20 knockdown was combined with TFIIS knockdown (Fig. 5A, siTCEA1+2+RNF20). Hence, the increased transcriptional elongation rate of the RNF20-suppressed genes after RNF20 depletion is dependent on the activity of TFIIS.

RNF20 binds RNA polymerase II through the hPAF1 subunit of the PAF1 complex (Kim et al., 2009). TFIIS binds directly to both RNA polymerase II and the hPAF1 complex (via the hPAF1 and hLEO1 subunits); the direct interaction between TFIIS and hPAF1C results in their cooperative binding to Pol II and a strong synergistic effect on transcriptional elongation (Kim et al., 2010). To assess whether RNF20 activity may impede transcriptional elongation by attenuating the TFIIS-hPAF1 interaction, we performed co-immunoprecipitation analysis. As seen in Figure 5B, the interaction between TFIIS and hPAF1 became more apparent upon RNF20 depletion (left panel; quantification of three independent experiments shown in the right panel). Moreover, depletion of RAD6A, which exerts similar effects on H2Bub levels and gene expression (Figure 1 D,E), likewise augmented the TFIIS-hPAF1 interaction (Figure 5C). Together, these results are strongly consistent with the conclusion that the catalytic activity of the RNF20-RAD6A complex, presumably through H2B ubiquitylation, impedes the hPAF1-TFIIS interaction.

TFIIS and RNF20 counter each other in the transcriptional response to EGF

RNF20 preferentially suppresses the expression of many EGF-inducible genes (Shema et al., 2008). To investigate a possible involvement of TFIIS in that context, we examined whether TFIIS is recruited to such EGF target genes upon induction by EGF. As shown in Figure 6A, this was indeed found to be the case; moreover, the kinetics of recruitment paralleled those of transcription (see Fig. 6B). In contrast, no increase in TFIIS binding was observed for *GAPDH* and *p21*, which are not transcriptionally induced by EGF (Figure 6A). Furthermore, depletion of TFIIS (TCEA1 and TCEA2) mildly but reproducibly attenuated the transcriptional response of those genes to EGF, as assessed by analysis of steady state mRNA (Figure 6B), as well as of heterogeneous nuclear RNA indicative of relative transcription rates (Figure 6C). Depletion of TCEA1 alone exerted similar effects (Supplemental Fig. S3A,B). Hence, recruitment of TFIIS to a subset of EGF target genes is necessary for their optimal transcriptional induction, implicating TFIIS as a positive

regulator of this response. Some EGF target genes are not suppressed by RNF20 (Shema et al., 2008). Interestingly, examination of representative genes from that group revealed that they are refractory to TFIIS ablation (Supplemental Fig. S3C), further supporting the specific dependence of RNF20-suppressed genes on TFIIS activity.

MCF10A cells are stimulated to migrate by addition of EGF, and depletion of RNF20 results in a robust increase in cell migration, consistent with its effects on EGF-induced gene expression (Shema et al., 2008). We therefore next asked whether the induction of cell migration by RNF20 depletion is also dependent on TFIIS. As expected, knockdown of RNF20 led to a marked increase in cell migration in response to EGF (Figure 7A). Knockdown of TFIIS alone (siTCEA1+2) caused a slight decrease in EGF-induced cell migration. Remarkably, concomitant depletion of TFIIS (siTCEA1+2+RNF20) abolished almost completely the stimulatory effect of RNF20 knockdown. Overall, these findings imply that the opposing effects of RNF20 and TFIIS on the expression of the RNF20-repressed genes have also biological consequences.

Discussion

RNF20, in complex with RNF40, is the main E3 ligase that targets histone H2B for monoubiquitylation. Despite the general association of H2Bub with transcription, only a subset of genes seem to be regulated by that modification (Shema et al., 2008). Here, we show that the E3 activity of RNF20 is essential for that regulation. This conclusion is based on the failure of a RING-deficient RNF20 mutant to rescue the effects of RNF20 knockdown, as well as the fact that depletion of RAD6A, which we find to be the principal E2 enzyme in that process, exerts the same transcriptional effects as depletion of RNF20. This is in agreement with the finding that both human RAD6 proteins (hRAD6A and hRAD6B), but not hUbcH6 which can also serve as an E2 enzyme for H2Bub (Kim et al., 2005; Zhu et al., 2005), fully complement the function of yeast RAD6 in H2B ubiquitylation (Kim et al., 2009). RAD6A comprises the majority of the endogenous RAD6 protein pool (Koken et al., 1996); thus, it is not surprising that its depletion leads to a marked decrease in H2Bub levels.

Significantly, our findings strongly implicate the elongation factor TFIIS in the mechanism of RNF20-mediated gene repression. In particular, we show that upon RNF20 depletion, RNF20-suppressed genes are upregulated only in the presence of TFIIS. Furthermore, overexpression of TFIIS alone leads to selective upregulation of RNF20-suppressed genes, while not affecting other genes. Interestingly, whereas TFIIS is recruited more effectively to all tested genes upon RNF20 depletion (Fig. 4C), only a minority of those genes (the RNF20-suppressed subgroup) are functionally dependent on the increased recruitment of TFIIS. Together, our results imply a novel mechanism for the regulation of a subset of genes by RNF20 and H2B ubiquitylation (Figure 7B). RNF20-suppressed genes reside within relatively closed chromatin, characterized also by high H2Bub content (Shema et al., 2008). According to the proposed model, the catalytic activity of the RNF20/RNF40/RAD6A, presumably through H2B ubiquitylation, interferes with TFIIS binding to the PAF1 complex, resulting in inefficient transcriptional elongation of genes that require TFIIS for this process. Upon RNF20 depletion, H2Bub levels are drastically reduced and TFIIS can therefore bind the PAF1 complex. The interaction between TFIIS and the PAF1 complex results in their cooperative binding to Pol II and a synergistic effect on transcriptional elongation (Kim et al., 2010), augmenting the accumulation of transcripts. For the majority of the genes that are not regulated by RNF20, however, the increased binding of TFIIS to Pol II upon RNF20 depletion has no effect on transcription, suggesting that these genes do not require TFIIS for optimal transcription elongation. The selective requirement of TFIIS by the RNF20-suppressed genes may be due to the fact that they reside within relatively

closed chromatin, which is presumably harder to transcribe. This conjecture is supported by the observation that the RNF20-suppressed genes are associated with high constitutive Pol II levels, despite being expressed at relatively low levels (Shema et al., 2008). TFIIS may therefore be specifically needed to reactivate the arrested elongation complexes on these genes. Indeed, we found that depletion of RNF20 specifically increased the elongation rate of these genes, in a TFIIS-dependent manner. Presumably, genes that are not dependent on RNF20 and reside in a more open chromatin context do not require TFIIS binding for optimal transcription elongation.

H2B is the main known target for the catalytic activity of the RAD6A-RNF20-RNF40 complex. It is therefore plausible that the transcriptional effects explored in this study are mediated by H2B ubiquitination. However, at present we can not formally rule out the possibility that some of those transcriptional effects are due to ubiquitylation of other proteins by that complex, or even that the complex exerts some unknown mode of transcriptional interference that requires the RING domain of RNF20 but does not rely on protein ubiquitylation. In yeast, such question can be addressed directly through the construction of strains carrying a point mutation in the ubiquitylation site of the single H2B protein (Henry et al., 2003; Tanny et al., 2007). Indeed, by applying this strategy to the fission yeast *S.pombe*, Tanny et al. found that the gene expression profile of cells carrying such point mutation exhibited substantial overlap with that of strains deleted for *BRL1* and *BRL2*, the *S.pombe* BRE1 orthologs. This suggests that *BRL1* and *BRL2* regulate transcription mostly through ubiquitylation of H2B. Nevertheless, the transcriptional profiles were not completely identical between the H2B mutant strain and the *BRL1+BRL2*-deficient strain. Hence, it was proposed that these E3 ligases likely have additional ubiquitylation targets that also contribute to gene regulation (Tanny et al., 2007). Unfortunately, this strategy cannot be implemented effectively in mammalian cells, whose genomes carry numerous H2B genes with nucleotide sequence differences that severely hinder the use of siRNA to knock all of them down (Marzluff et al., 2002).

Our findings are reminiscent of earlier observations with regard to the negative elongation factor (NELF), which has been shown to inhibit TFIIS binding to Pol II, presumably by a competition model (Palangat et al., 2005); raising TFIIS concentration gradually overcame this inhibition. It is noteworthy that a recent study, performed in embryonic stem cells, found over 90% of the genes to be regulated at the elongation level by promoter-proximal pausing, mediated by the negative elongation factors NELF and DSIF (Rahl et al., 2010). Stalling of Pol II, bound to NELF, mediates cytokine gene expression (Adelman and Rogatsky, 2010). Interestingly, the induction of the cytokine IL-8 in response to EGF signaling is suppressed by RNF20 (Shema et al., 2008). Here we show that TFIIS is required for optimal induction of IL-8, demonstrating the opposing effects of RNF20 and TFIIS in its transcriptional regulation.

Interestingly, c-Myc, shown here to be regulated by RNF20 and TFIIS, was reported to play a key role in releasing paused Pol II, by recruiting the positive elongation factor P-TEFb (Rahl et al., 2010). This may therefore contribute to a positive feedback mechanism whereby, upon downregulation of RNF20 or signal-dependent attenuation of its catalytic activity, TFIIS-dependent c-Myc induction further enhances transcriptional elongation of paused genes; similar effects may be exerted by increased activity of H2Bub-specific deubiquitinating enzymes (DUBs) (Zhang et al., 2008). It will be of great interest to gain further insight into the intricate interplay between positive and negative elongation factors, as well as the signals and cellular contexts that modulate this interplay to selectively regulate specific gene expression programs.

Finally, our findings may also pertain to human cancer. The misregulation of a variety of histone modifications can actively contribute to cancer (Chi et al., 2010). RNF20 has attributes of a putative tumor-suppressor, chiefly through selective suppression of pro-oncogenic genes that promote proliferation (e.g. *c-Myc*, *c-Fos*) along with transcriptional activation of tumor suppressors such as p53 (Espinosa, 2008; Shema et al., 2008). TFIIS, on the other hand, was proposed to have a positive link to cancer: its depletion in cancer cell lines was shown to inhibit cell proliferation and induce apoptosis (Hubbard et al., 2008). In addition, homozygous null mutants for TFIIS show a dramatic increase in apoptotic cells in the liver (Ito et al., 2006). This is partly due to the fact that TFIIS directly upregulates the transcription of the *Bcl-XL* gene, which encodes a potent anti-apoptotic protein implicated in the survival of cancer cells (Ito et al., 2006; Nagata et al., 2009). Remarkably, examination of the Oncomine database (www.oncomine.org) reveals significant upregulation of TCEA1 expression in a variety of human tumors (Supplemental Figure S4). It is also noteworthy that the *TCEA2* gene resides within a region of chromosome 20 that is frequently amplified in cervical cancer, and *TCEA2* mRNA is often overexpressed in these tumors, giving rise to the suggestion that it may play a role in cervical carcinogenesis (Scotto et al., 2008). Indeed HeLa cells, derived from a cervical carcinoma, express substantial amounts of *TCEA2* mRNA, and knockdown of TCEA2 alone is already sufficient to yield measurable transcriptional effects in these cells (Supplemental Fig. S2D). Thus, excessive TFIIS activity may promote cancer, at least in some contexts.

In sum, our results strongly suggest that part of the tumor suppressor activities of RNF20 may be mediated via inhibition of TFIIS binding, with consequent downregulation of cancer-promoting genes whose transcriptional elongation relies on TFIIS. This calls for further analysis of the interplay between TFIIS and RNF20 in human cancer.

Experimental procedures

Cell culture and transfections

Human cervical carcinoma HeLa cells were grown in DMEM with 10% bovine serum supplemented with antibiotics, and MCF10A human mammary epithelium-derived cells were grown in DMEMF12 medium with 5% heat-inactivated horse serum, supplemented with antibiotics and 10 µg/ml insulin, 0.1 µg/ml cholera toxin, 0.5 µg/ml hydrocortisone and 10 ng/ml EGF. SMARTpool oligonucleotides for siRNA transfection were purchased from Dharmacon. Transfections were carried out with Dharmafect 1 (HeLa) or Dharmafect 4 (MCF10A) reagent according to the manufacturer's protocol. The siRNA sequence targeting the 5' UTR of *RNF20* mRNA was CTGCACGGGCCTTGGAAAA. Transfection of expression plasmids was carried out using the jetPEI DNA transfection reagent (101-10, Polyplus Transfection), according to the manufacturer's protocol.

Antibodies

The following commercial antibodies were used: anti-H2B (07-371, Millipore); Anti-Pol II (ab5408, Abcam), Anti-RNF20 (ab32639, Abcam), Anti-Paf1 (Western-blot: A300-172A, IP: A300-173A, Bethyl Laboratories), Anti-GAPDH (MAB374, Millipore), and Anti-flag (F3165, Sigma). Anti-H2Bub is described in Minsky et al. (Minsky et al., 2008). Anti-TFIIS is described in Kim et al. (Kim et al., 2010).

RNA purification and quantitative Real-Time PCR

For Real-time reverse transcription (RT-PCR) analysis, total RNA was extracted with the NucleoSpin kit (Macherey Nagel, Germany). One microgram of each RNA sample was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) and random hexamer primers (Applied Biosystems). Real-time PCR was

performed in a StepOne real-time PCR instrument (Applied Biosystems, Foster City, CA) with SYBR Green PCR Supermix (Invitrogen). The primers used are listed in Supplemental table 1.

Chromatin immunoprecipitation (ChIP)

ChIP analysis was performed as described (Minsky and Oren, 2004). The primers used are listed in Supplemental table 1.

Fractionation of chromatin-bound proteins

HeLa cells were harvested and washed twice with ice-cold PBS, then incubated on ice for 5 minutes with 100mM NaCl, 300mM sucrose, 3mM MgCl₂, 10mM Pipes pH 6.8, 1mM EGTA, 0.2% Triton X-100 and protease inhibitor mix (Sigma). Cells were centrifuged, and the supernatant was collected as the “unbound” fraction. The remaining “bound” fraction was washed with PBS and then collected in 100mM NaCl, 300mM sucrose, 3mM MgCl₂, 10mM PIPES pH 6.8, 1mM EGTA and 0.5U/μl DNaseI, supplemented with protease inhibitor mix (Sigma).

Co-immunoprecipitation (co-IP) analysis

HeLa cells were harvested, washed with ice-cold PBS and lysed on ice in NET lysis buffer (50mM Tris-HCl, pH7.4, 150mM NaCl, 1mM EDTA 0.1% NP40) supplemented with protease inhibitor mix (Sigma). Cells were sonicated in a Bioruptor sonicator (Diagenode) for a total of 5 minutes, including intervals of 30 seconds on and 1 minute off, and then centrifuged at 13000RPM for 10 minutes at 4°C. Total protein content was determined with the BCA protein assay kit (PIERCE, #23227). Anti-flag antibodies (A2220, Sigma), covalently attached to beads, were washed once with ice-cold PBS and twice with NET buffer. Equal amounts of protein from each sample were incubated with the antibody beads at 4°C overnight. Next, beads were washed three times with NET buffer, and elution was carried out using flag peptide (F3290, Sigma) in PBS. Samples were resolved by SDS-PAGE.

Cell migration assay

Cell migration analysis was as described (Katz et al., 2007).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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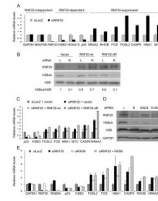


Figure 1. The E3 ligase activity of RNF20 is necessary for its regulation of gene expression

(A) qRT-PCR analysis of RNF20-independent, RNF20-dependent and RNF20-suppressed genes, 48 hours after transient transfection of HeLa cells with RNF20 siRNA (siRNF20) or LacZ siRNA (siLacZ). Also shown is *RNF20* itself, as a measure of knockdown efficiency. Gene values were divided by the control siLacZ samples, which were set as 1. Prior to the division, values were normalized to *GAPDH* mRNA in the same sample, except for the bars indicating the raw values of *GAPDH* itself. Bars indicate averages of values from duplicate qPCR reactions; error bars represent standard deviation. Similar data was obtained in 10 independent experiments. See Supplemental Fig. S1A,B for validation with 3 different RNF20 siRNA oligonucleotides.

(B) HeLa cells were transiently transfected with siRNA oligonucleotides directed to the 5' UTR of endogenous RNF20 (R) or with siLacZ (L). 24 hours later, cells were transfected with expression plasmids encoding either wild-type RNF20 (RNF20-wt) or a delta RING mutant thereof (RNF20-dR), both lacking the 5' UTR of RNF20 mRNA and therefore resistant to the siRNA; empty vector was transfected as control. Cells were harvested 48 hours later for Western blot analysis with the indicated antibodies. The relative abundance of H2Bub, corrected for total H2B in the same sample, is shown at the bottom; the H2Bub/H2B ratio in the control sample (first lane) was taken as 1.0.

(C) qRT-PCR analysis of RNF20-dependent genes (*H2BD* and *p53*) and RNF20-suppressed genes (*FOSL2*, *FOS*, *HBA1*, *MYC*, *CASP9* and *NR4A2*), from the experiment described in (B). Values and error bars were calculated as in (A). Similar data was obtained in 3 independent experiments.

(D) Western blot analysis of RNF20 and H2Bub 48 hours after transient transfection of HeLa cells with siLacZ (L), siRNF20 (R), siRAD6A (RAD6) or a combination of siRNF20 and siRAD6A (R+RAD6). GAPDH and H2B serve as loading controls.

(E) qRT-PCR analysis of the experiment described in (D) for the RNF20-independent gene *GAPDH*, the RNF20-dependent genes *H2BD* and *p53*, and RNF20-suppressed genes *FOSL2*, *FOS*, *HBA1*, *CASP9*, *RHOB* and *NR4A2*. Also shown are *RNF20* and *RAD6A*, as a measure of knockdown efficiencies. Values and error bars were calculated as in (A). Similar data was obtained in 3 independent experiments.

See Supplemental Fig. S1C, D for analysis of RNF40 depletion.

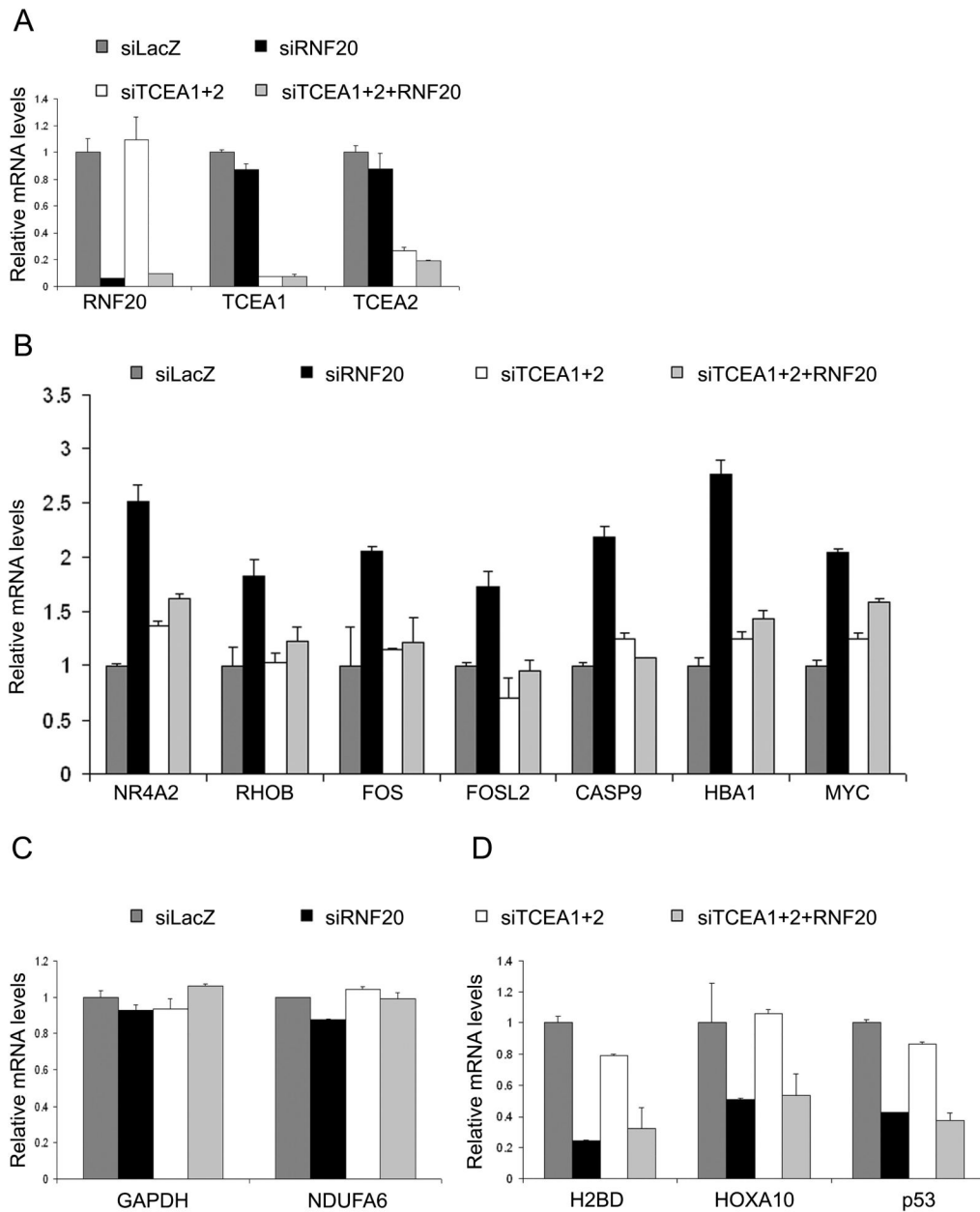


Figure 2. TFIIIS depletion abolishes the increased transcription of RNF20-suppressed genes following RNF20 knockdown

(A) qRT-PCR analysis of *RNF20*, *TCEA1* and *TCEA2* 48 hours after transient transfection of HeLa cells with the indicated siRNA oligonucleotides. Values and error bars were calculated as in Fig. 1A. Similar data was obtained in 6 independent experiments. See also Supplemental Fig. S2A for Western blot analysis of the same experiment.

(B-D) qRT-PCR analysis of RNF20-suppressed genes (B), RNF20-independent genes (C) and RNF20-dependent genes (D) 48 hours after transfection as in (A). Values and error bars were calculated as in Fig. 1A. Gene values were divided by the control siLacZ samples, which were set as 1. For (B) and (D), prior to the division, values were normalized to *GAPDH* mRNA. (C) depicts the raw values of *GAPDH* and *NDUFA6* mRNA, used for normalization of the other values. Similar data was obtained in 6 independent experiments.

See also Supplemental Fig. S2B for analysis of heterogeneous nuclear RNA of RNF20-suppressed genes, and Supplemental Fig. S2C-F for the effects of individual knockdown of either TCEA1, TCEA2 or TCEA3.

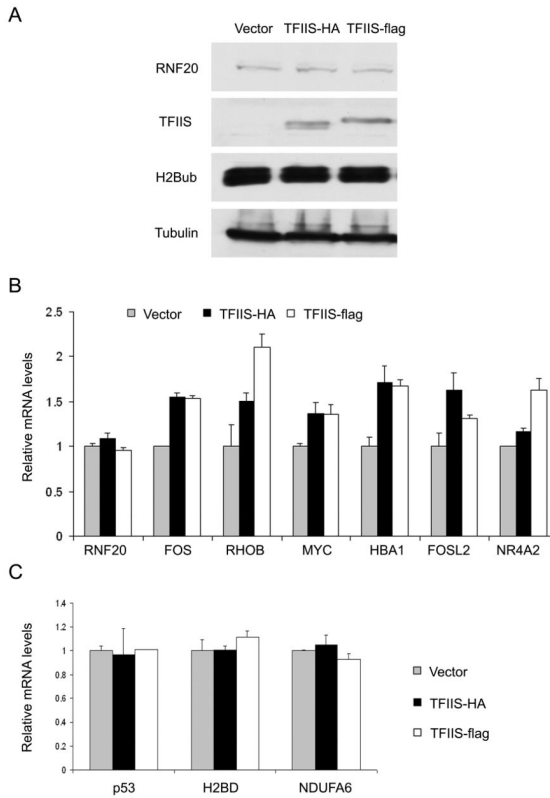


Figure 3. TFIIIS overexpression increases the transcription of RNF20-suppressed genes

(A) Western blot analysis of RNF20, TFIIIS and H2Bub 48 hours after transient transfection of HeLa cells with control empty vector, HA-tagged TCEA1 or flag-tagged TCEA1 (TFIIIS-HA and TFIIIS-flag, respectively). Tubulin served as a loading control.

(B) qRT-PCR analysis of RNF20-suppressed genes, from the experiment described in (A). Also shown is *RNF20* mRNA. Values and error bars were calculated as in Fig. 1A. Similar data was obtained in 3 independent experiments.

(C) qRT-PCR analysis of the RNF20-dependent genes *p53* and *H2BD* and RNF20-independent gene *NDUFA6*, from the experiment described in (A). Values and error bars were calculated as in Fig. 1A. Similar data was obtained in 3 independent experiments.

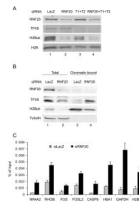


Figure 4. RNF20 knockdown increases the occupancy of TFIIS on chromatin

(A) Western blot analysis of RNF20, TFIIS, H2Bub and H2B 48 hours after transient transfection of HeLa cells with the indicated siRNA oligonucleotides. Equal amounts of total protein were loaded in each lane.

(B) HeLa cells were transiently transfected with the indicated siRNA oligonucleotides. 48 hours later, the chromatin bound fraction was isolated as described under Experimental procedures, and subjected to Western blot analysis with the indicated antibodies. Whole cell extract aliquots (Total) were analyzed in parallel.

(C) Chromatin immunoprecipitation (ChIP) analysis of relative TFIIS abundance on RNF20-suppressed genes (*NR4A2*, *RhoB*, *FOS*, *FOSL2*, *CASP9*, *HBA1*), the RNF20-independent gene *GAPDH* and the RNF20-dependent gene *H2BD*, in siLacZ and siRNF20-transfected HeLa cells. Immunoprecipitated DNA and input DNA were quantified by qPCR with primers specific for the 5' transcribed regions of the indicated genes. Results are presented as percentage of input. Bars indicate averages of data from duplicate qPCR reactions; error bars represent standard deviation. Similar data was obtained in 3 independent experiments.

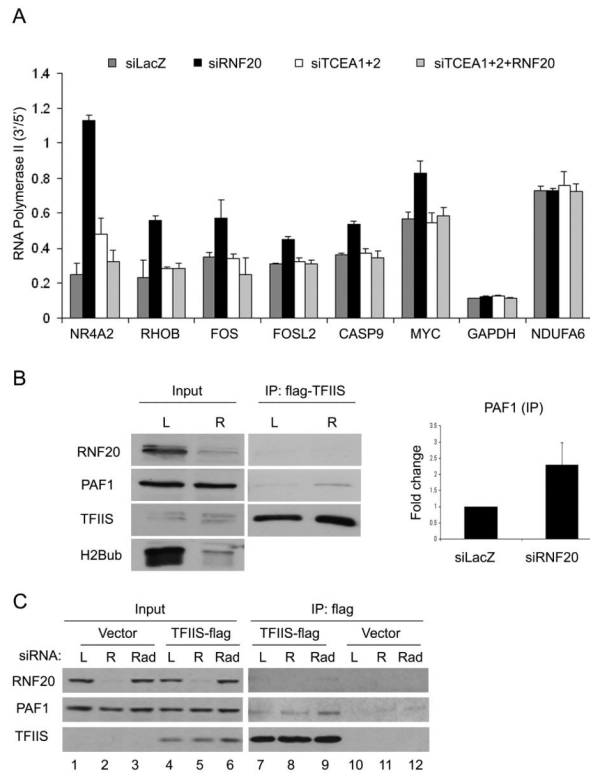


Figure 5. Downregulation of RNF20 facilitates the elongation of RNF20-suppressed genes and increases the interaction between TFIIS and the PAF1 complex

(A) Ratio between RNA polymerase II occupancy near the 3' end versus near the 5' end of RNF20-suppressed genes (*NR4A2*, *RhoB*, *FOS*, *FOSL2*, *CASP9*, *MYC*) and RNF20-independent genes (*GAPDH* and *NDUFA6*). HeLa cells were transiently transfected with siLacZ, siRNF20, siTCEA1+2 or siTCEA1+2+siRNF20. 48 hours later, cells were subjected to ChIP analysis with antibodies against Pol II, followed by qPCR analysis with primers corresponding to the 5' and 3' ends of each of the transcribed regions. Bars indicate averages of data from duplicate qPCR reactions; error bars represent standard deviation. Similar data was obtained in 3 independent experiments.

(B, C) HeLa cells were transiently transfected with RNF20 (R) or LacZ (L) siRNA oligonucleotides in (B), or with RAD6A (Rad) siRNA oligonucleotides in (C). 24 hours later, cells were transfected with flag-tagged TCEA1 (TFIIS-flag) or empty vector as control. After an additional 48 hours, cells were harvested for immunoprecipitation (IP) with anti-flag antibodies; 3% of the total cell extract served as input. The IP and input samples were subjected to Western blot analysis with the antibodies indicated on the left. The graph in B represents quantification of the hPAF1 signal in the indicated IP samples, derived from three independent experiments (including the one shown in B). The hPAF1 signal in the siLacZ sample for each experiment was set as 1, and the fold increase in the corresponding siRNF20 sample was calculated accordingly. Quantification was with ImageJ. Error bars represent standard deviation.

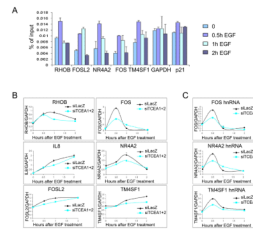


Figure 6. TFIIIS is recruited to EGF targets, and counters RNF20 in the transcriptional response to EGF

(A) ChIP analysis of relative TFIIIS abundance in HeLa cells stimulated with EGF (20 ng/ml) for 0, 0.5 hours, 1 hour or 2 hours. Prior to EGF addition, cells were serum starved for 16 hours. Immunoprecipitated DNA and input DNA were quantified by qPCR with primers for the EGF target genes *RHOA*, *FOSL2*, *NR4A2*, *FOS* and *TM4SF1*, and also for *GAPDH* and *p21*, which are not responsive to EGF. Bars indicate averages of data from duplicate qPCR reactions; error bars represent standard deviation. Similar data was obtained in 3 independent experiments.

(B,C) qRT-PCR analysis of total mRNA (B) or heterogeneous nuclear precursor RNA (C) levels of the indicated EGF target genes at various times after exposure to EGF of HeLa cells transiently transfected with TCEA1+TCEA2 siRNA (light blue) or LacZ siRNA (black). All values are normalized to *GAPDH* mRNA. Precursor RNA levels were measured with primer pairs derived from intronic sequences of the indicated genes. Values indicate averages of data from duplicate qPCR reactions; error bars represent standard deviation. Similar data was obtained in 4 independent experiments. See also Supplemental Fig. S3 for parallel analysis after TCEA1 knockdown alone.

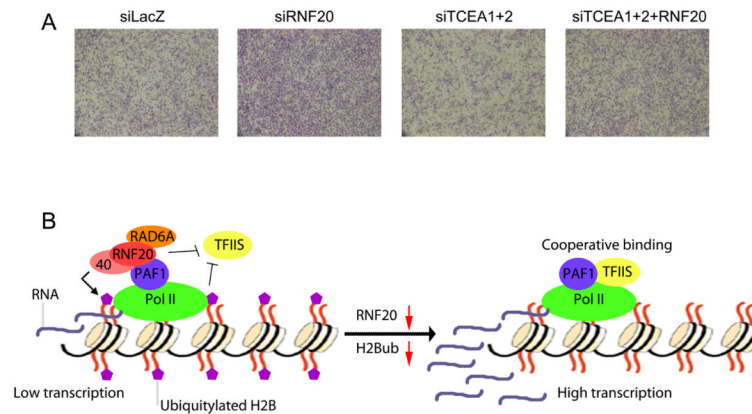


Figure 7. Enhancement of EGF-induced cell migration by RNF20 depletion is dependent on TFIIIS

(A) MCF10A cells were transiently transfected with siRNA oligonucleotides for LacZ (siLacZ), RNF20 (siRNF20), TFIIIS (siTCEA1+2) or a combination of TFIIIS and RNF20 (siTCEA1+2+RNF20). 48 hours later, 70,000 cells were plated in the upper compartment of a 24-well Transwell tray (Corning), and allowed to migrate for 16hr towards EGF (added to the bottom compartment). Migrating cells were visualized by staining the membrane with methyl violet, followed by photography under a binocular. See also Supplemental Fig. S4 for analysis of TCEA1 mRNA expression in human tumors.

(B) Proposed model for the regulation of a subset of genes by RNF20 and H2Bub. RNF20-suppressed genes are associated with high levels of H2Bub (purple), and transcribed with low efficiency owing to inefficient elongation. Under those conditions, binding of TFIIIS is inhibited, either by H2Bub or/and via additional activities of the RNF20/RNF40/RAD6A complex. Upon RNF20 depletion, H2Bub levels decline, enabling recruitment of TFIIIS to the chromatin and cooperative binding of TFIIIS to the PAF1 complex and to Pol II. This leads to an increase in transcription rates. The chromatin cartoon was adapted from Marmorstein (Marmorstein, 2001).