Transcription elongation factor ELL2 directs immunoglobulin secretion in plasma cells by stimulating altered RNA processing

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Abstract

Immunoglobulin secretion is modulated by a competition between use of a weak promoter proximal poly(A) site and a non-consensus splice site in the last secretory-specific exon of the heavy chain pre-mRNA. RNA polymerase II transcription elongation factor ELL2, induced in plasma cells, enhanced both polyadenylation and exon skipping with the *Igh* gene and reporter constructs. Lowering ELL2 expression by hnRNP F transfection or siRNA reduced secretory-specific forms of IgH mRNA. ELL2 and polyadenylation factor CstF-64 co-tracked with RNA polymerase II across the *Igh* mu and gamma gene segments; association of both factors was blocked by ELL2 siRNA. Thus loading of ELL2 and CstF-64 on RNAP-II was linked, causative for enhanced proximal poly(A) site use and necessary for IgH mRNA processing.

Keywords

Polyadenylation; Splicing; transcription elongation; *Igh* expression; B cell

INTRODUCTION

Understanding how the pre-mRNA for IgH is processed is key for understanding how secreted immunoglobulin (Ig) is made. IgH mRNA processing is also a well-recognized model for understanding how the splicing and polyadenylation reactions compete in a primary transcript1 since it has been estimated that as many as 20% of all human genes may contain competing RNA splicing and polyadenylation signals2. In plasma cells the secreted form of the Ig protein is produced by the use of the promoter-proximal, weak secretory-

specific Ig poly(A) site in the mRNA. Memory or mature B cell mRNA encodes the membrane-bound B cell receptor for antigen, produced by use of the weak non-consensus 5' splice site, embedded in the secretory-terminal exon, and downstream membrane exons; thus resulting in usage of the strong promoter-distal IgH membrane poly(A) site. The molecular mechanisms that influence the RNA processing choice critically impact the immune response and expression of secreted Ig, the primary goal of plasma cell development.

In plasma cells versus mature B cells the *Igh* transcription start site region exhibits increased binding of modified RNA polymerase II (RNAP-II) harboring phosphorylated Ser-2 and Ser-5 in its carboxyl-terminal domain (CTD), increased loading of polyadenylation factors CstF and CPSF onto the RNAP-II, as well as increased binding of ELL2, a transcriptional elongation factor; these changes accompany the shift to the use of the promoter-proximal secretory poly(A) site[3]. The binding of ELL2 and CstF-64 to RNAP-II is dependent on phosphorylation of Ser-2 on the CTD of RNAP-II[3]. No changes occur in histone H3 K9, K14 acetylation[3], consistent with the minimal changes in transcriptional activation of the *Igh* locus previously observed between the cell types[4].

Factors critical to correct processing of the IgH pre-mRNA are expected to be differentially expressed in plasma cells and may interact directly or indirectly with the *Igh* locus; ELL2 fulfills these criteria. The ELL (eleven-nineteen lysine-rich leukemia protein) family of factors acts *in vitro* in a positive manner to promote elongation by keeping the 3'OH of nascent RNA in alignment with the catalytic site, thus preventing RNAP-II backtracking[5–8]. The shift to the secretory specific form of IgH mRNA in plasma cells is preceded by up-regulation of the transcription factors Blimp-1 and IRF4; Blimp-1 targets include the genes encoding ELL2 and its associated factor EAF2 (refs. 9, 10). ELL2 mRNA is also induced by high expression of IRF4 in multiple myeloma where IRF4 binds to the *ELL2* promoter[11]. It has been demonstrated in several other genes that varying the rate of RNAP-II elongation by mutation or drugs can influence splicing patterns[12]. Cdc73, a component of the constitutive, multi-protein, polymerase-associated elongation factor, was recently shown to facilitate polyadenylation and enhance promoter proximal poly(A) site use[13].

In this study we establish a mechanistic role for the elongation factor ELL2 in regulating IgH pre-mRNA processing. ELL2 was down-modulated in a plasma cell line by transfection with heterogenous ribonucleoprotein (hnRNP) F, a condition that correlates with reduced production of the mRNA encoding the secreted form of IgH[14]. Proteins of 58 kDa and 59 kDa arise from cleavage of the full-length 80 kDa ELL2 or from internal initiation of translation at M_{186}; the abundance of the shorter ELL2 proteins was increased after lipopolysaccharide (LPS) stimulation, a condition that promotes Ig secretion. ELL2 depletion by specific shRNA reduced the association of CstF-64, a polyadenylation factor with the RNAP-II on the *Igh* promoter in plasma cells. ELL2 accelerated the use of the secretory-specific poly(A) site in the *Igh* gene, both for γ2band μ isotypes. Separating RNA processing into its component reactions, both the full-length and the smaller ELL2 proteins were able to stimulate promoter-proximal secretory poly(A) site choice and exon skipping. Thus ELL2 uniquely influences IgH pre-mRNA processing by both enhancing exon skipping of the non-consensus splice site and facilitating use of the weak promoter proximal poly(A) site.

RESULTS

Modulation of ELL2 mRNA with hnRNP F transfection

The binding of hnRNP F to G-rich sequences (GRS) in pre-mRNA reduces access of the polyadenylation machinery to a poly(A) site15 and modulates splicing16. The abundance of hnRNP F protein is high in B cells where membrane forms of IgH mRNA predominate while hnRNP F expression is lower in plasma cells where there is an excess of secretory-specific mRNA (Fig. 1a)14. To look at downstream targets, we performed microarray analyses on the mRNA expressed in hnRNP F transfected AxJ plasma cells14 and in controls of AxJ cells17 transfected by the vector lacking hnRNP F and in the A20 B cell line. Of the 207 genes whose expression differed by more than 2-fold between A20 B cells and AxJ cells only three genes had lower expression in both mature B cells and hnRNP F-transfected AxJ cells as compared to AxJ plasma cells; therefore these genes might be important for plasma cell differentiation and Ig secretion. Those mRNAs encoded ELL2 (103892_r_at), integrin α_4 (102655_at), and retinoblastoma-like p107 (104476_at). ELL2 is a transcription elongation factor a map of which is shown schematically in Fig. 1b. The microarray data for those mRNAs were confirmed by reverse transcriptase plus quantitative PCR (RT-qPCR) (Table 1) using primers listed in Supplementary Table 1 online. As determined by using the GRS finder (http://bioinformatics.ramapo.edu/QGRS2/index.php) the three genes modulated by hnRNP F overexpression in plasma cells contained an unusually high number of potential hnRNP F target sites: *Ell2* has 201 G-rich sites; *Rbl1* has 432; and *Itga4* has 127 potential interaction sites. This high GRS-content is in contrast to several housekeeping genes *Actb (β actin)*, *Gapdh* (glyceraldehyde 3-phosphate dehydrogenase) or *Hprt1* (hypoxanthine phosphoribosyltransferase) where only a small number of G-rich sites were found. It appears that ELL2 was susceptible to regulation by hnRNP F and was down-regulated in B cells where hnRNP F expression is high.

To rule out the possibility of differentially expressed, alternatively spliced forms of ELL2 we performed RNA hybridization experiments. The size of the ELL2 mRNA was verified by RNA blot to be a single ~4 kb mRNA in either A20 or AxJ cells (Fig. 2a, lanes 3, 4 and 7, 8). PC4 is a transcription factor that is also expressed to a higher extent in plasma cells (Fig. 2a, lanes 1, 2, 5, 6). GAPDH mRNA was used as a size control (Fig. 2a, lanes 5–8). We confirmed this result by applying a systematic PCR approach with overlapping sets of primers. The observed PCR products revealed no evidence for other mRNA species in either A20 or AxJ cells (data not shown).

ELL2 protein expression

To examine ELL2 protein expression in mature B cells and plasma cells we generated antibodies that recognize ELL2. Rabbit antibodies R4213 was generated to peptides corresponding to internal amino acids of ELL2 (Fig. 1b). The R4213 antibody reacted with the protein made from a transfected full-length ELL2 cDNA clone that was identified by a cloned epitope tag (Supplementary Fig. 1 online). When immunoblots were probed with the R4213 antibody, virtually no full-length ELL2 protein was detectible in cells or nuclei however expression of a 58–59 kDa protein doublet was much more abundant in AxJ plasma cells than in A20 B cells (Fig. 2b, lanes 1, 2 and Supplementary Fig. 1). The

antibody R4502, to a different ELL2 epitope (Fig. 1b), described previously[3], showed a similar pattern of protein expression to that seen with R4213. Immunoblot analysis of ELL2 protein expression in mouse mature B cells versus plasma cells with the 83104 monoclonal antibody that recognizes the NH2-terminal end of bacterial produced ELL2[7] revealed no change in expression of the predicted full-length ELL2 protein species (Fig. 2b, lanes 3, 4 with darker exposure in lanes 5, 6). Full-length ELL2 protein must therefore be in low abundance, turn over rapidly, or both. The more rapidly migrating proteins recognized by the secondary antibody in the immunoblots are the IgH membrane form, which serves as a loading control, and the IgH secretory form; the increase in the secretory form confirms that the AxJ cells behave as plasma cells.

In addition, the 58–59 kDa form of ELL2, but not the 80 kDa form, was increased in mouse splenic B cells that had undergone 72 h of stimulation with LPS *in vitro* (Fig. 2c), a time point at which Blimp-1, Ig secretion and Ig light chain (Fig. 2c, lane 2) was induced. We confirmed that the 58–59 kDa band was indeed ELL2 protein by *in vitro* translation of the cDNA (Supplementary Fig. 2 online) and by use of shRNA specific for ELL2 (iell2) which diminished expression of the 59–58 kDa protein (Fig. 2d, lane 2). The results using untreated cells versus non-specific shRNA (iNS) were identical (data not shown).

An examination of the predicted ELL2 protein sequence across several species (Supplementary Table 2 online) disclosed numerous, evolutionarily conserved AUG methionine codons where translation might initiate, as well as regions where there may be cleavage of the protein at conserved RK sequences by stress-induced proteases[18]. Through a series of *in vitro* transcription-translation and site-directed mutagenesis experiments (Supplementary Fig. 2), we show that the shorter forms of ELL2 protein arise from both post-translational processing (59 kDa), most likely from the multiple tryptic cleavage sites near M_{186}, and from initiation of protein synthesis at M_{186} (the 58 kDa form). The amino acid conservation in this region across many species indicates a potentially important role for these two processes in ELL2 function.

ELL2 functionally associates with the *Igh* gene

We had previously shown more ELL2 and polyadenylation factors including CstF-associated with RNAP-II at the *Igh* promoter in AxJ plasma cells than in A20 B cells[3], implying B cell stage-specific differences in the ability to processing mRNA. This association with polymerase was inhibited by 5,6-dichlorobenzimidazole (DRB), an adenosine analog that inhibits Ser-2 addition to the CTD of RNAP-II; the observed linkages with the polymerase are therefore dependent on the phosphorylation state of the CTD[3]. Cleavage stimulation factor CstF-64 recognizes a GU or U-rich site ~50 nucleotides downstream of the AAUAAA and induces cutting of the pre-mRNA, one of the final steps prior to 3′ poly(A) addition. To determine if association of ELL2, CstF-64 and RNAP-II occurred in splenic B cells, we performed chromatin immunoprecipitations (ChIP) with antibodies before and after 72 h of stimulation with LPS, a condition in which B cells are activated and Ig secretory mRNA production is induced. The numerous V_H regions expressed in splenic B cells precluded determination of the protein-protein linkages at the promoter, but it is clear that RNAP-II, ELL2, and CstF-64 associate with the *Igh* μ gene

segment across its length from J_4 onward, after LPS treatment (Fig. 3a) and that the relative amount of ELL2 versus CstF-64 does not vary significantly even 3′ from the secretory poly(A) site.

Assessing the association of RNAP-II with ELL2 and CstF-64 across an IgG2b reporter gene construct in the mouse plasma cell line, J558L, would determine the extent of retention of these factors relative to the promoter. J558L cells lack an endogenous *Igh* gene but process the mRNA from a transfected IgG2b gene construct, driven by the *Igh* promoter, in a plasma cell manner19; expression of plasma cell markers like ELL2, Blimp-1 and Xbp-1 are high in these cells. Chromatin IP experiments with untransfected J558L and the *Igh* probes used for qPCR3 showed no signal greater than background (data not shown). The profiles of the RNAP-II, ELL2 and CstF-64 immunoprecipitated across this transfected IgG2b heavy chain gene segment are shown in Fig. 3b. The ratio of factors was relatively constant across the gene with a decrease in all the factors at the internal heavy chain enhancer, a site where the polymerase may travel faster or be less accessible to immunoprecipitation and a slight fall-off at the region after the most 3′ poly(A) site.

We have previously shown that ELL2 and CstF-64 are both associated with the phosphorylated form of the RNAP-II CTD3. If ELL2 were necessary for CstF-64 to bind to RNAP152 II, then removal of ELL2 should result in a reduction of CstF-64 binding. Indeed, J558L cells transfected with the IgG2b reporter plus a plasmid encoding siRNA to ELL2 (iell2) have less ELL2 as well as less CstF-64 bound to the TATA transcription start site region compared to cells transfected with reporter only or reporter plus nonspecific siRNA (iNS) (Fig. 4). Adding back either the WT ELL2 (wild type ELL2 with HA tag) or COOH ELL2 (encoding the 58 kDa ELL2 protein with a short N-terminal sequence tag), clones shown diagrammatically in Fig. 1b, rescued the binding of both factors to RNAP-II. We conclude that ELL2 facilitates recruitment of the polyadenylation factor CstF-64 to the RNAP-II near the *Igh* promoter. This binding is expected to favor promoter-proximal poly(A) site use, leading to more production of the IgH secretory-specific form in plasma cells versus B cells because the polymerase is poised to deliver the polyadenylation factors to the nascent RNA. Only a small fraction of the J558L cells become transfected with the reporter and shRNAs so the endogenous genes *Actb* and *Gapdh* in the surrounding cells serve as controls for equal immunoprecipitations with the various antibodies (see Supplementary Fig. 3 online). In those experiments *Actb* and *Gapdh* promoters are also associated with ELL2 and CstF-64 but to a lesser extent than the *Igh* gene.

ELL2 influences *Igh* precursor mRNA processing choice

To determine if ELL2 influences IgH mRNA processing, we performed a series of transient co-transfection experiments in the B cell line (A20) where ELL2 expression is normally low using the same IgG2b reporter construct as in the ChIP experiments (above) together with cDNAs for ELL2 and other factors. The ELL2 clones are illustrated diagrammatically in Figure 1b. After 48 h, the secretory and membrane IgG2b species produced in the transfected cells were quantified by RT173 qPCR. Equal efficiency of transfection by the ELL2 derivatives, CstF-64 and PC4 plasmids was determined by RT-qPCR of their mRNAs using primers unique for the transfected products (data not shown).

Setting the ratio of secretory:membrane mRNA produced from the IgG2b reporter plus empty vector in the B cells as 1, we saw (Fig. 5a) an enhancement in secretory specific mRNA production of approximately 4.8-fold when A20 cells were transfected with WT ELL2 (able to produce 80, 59 and 58 kDa forms). When cells were transfected with an ELL2 plasmid carrying the $M_{133, 138, 186}I$ mutation (able to produce 80 and 59 kDa forms), the production of IgG2b secretory mRNA from the reporter was still stimulated relative to empty vector, about 3.3-fold, almost as high as seen with the wild-type cDNA for ELL2. Based on these observations we conclude that ELL2 has a positive effect on influencing processing of the *Igh* gene products towards Ig secretion.

No significant stimulation of the secretory form over the membrane form was achieved with either the NH2 ELL2 or COOH ELL2, cloned regions of ELL2. The COOH ELL2 result was unexpected based on its ability to restore CstF-64 association with RNAP-II as shown previously in Fig. 4 using the same reporter with the *Igh* promoter. It is conceivable that the NH2 terminal epitope tag on COOH ELL2 may be interfering with its action or the missing portion may be important in the context of the totality of processing reactions. A plasmid encoding shRNA specific for the 3′ end of endogenous mouse ELL2 mRNA (iell2) resulted in 3-to 5-fold drop in the secretory to membrane ratio in the mRNA produced from the *Igh* reporter (Fig. 5a). A non-specific shRNA construct (iNS), with no complementarity to or effect on ELL2 expression, was ineffective at changing the secretory to membrane poly(A) mRNA choice. Adding back a plasmid encoding WT ELL2, restored IgH secretory mRNA production (iell2 + ELL2) to a significant extent. This is further support for the role of ELL2 in influencing IgH mRNA processing.

This stimulation of secretory mRNA with ELL2 was more efficient than cotransfection with CstF-64 alone (Fig. 5a). A dominant-negative mutation of CstF-64 (64 D/N), which lacks the final 282 amino acids at the COOH domain and are essential for CstF-64 function[20, 21], suppressed secretory poly(A) site use to about 12% that of empty vector. No enhancement of secretory-specific mRNA production was observed in transfections with PC4, a factor previously reported to have multiple transcription stimulatory activities including binding CstF-64 (refs. 22, 23).

To confirm that ELL2 stimulates secretory-specific poly(A) site use in the *Igh* locus in primary cells, mouse splenic B cells were isolated (t_o), incubated with LPS for 24 h, then transfected with plasmids encoding shRNAs (iNS or iell2, as described above) or mock-transfected. All cells were transfected with a plasmid carrying the green fluorescent protein marker (thus are GFP[+]). Twenty-four hours later (t48), the GFP^{+} cells were harvested. We set HPRT1 expression, previously shown to remain unchanged upon B cell terminal differentiation, as the RT-qPCR baseline[24]. Endogenous ELL2 relative expression rose from initially ~30% that of HPRT in unstimulated B cells to ~2.3 fold higher than HPRT in LPS-stimulated cells, approximately a 7-fold increase, a difference equivalent to that seen in the AxJ plasma cells (compare Table 1, Fig. 2c, and Fig. 5b). The secretory:membrane ratio rose in parallel following LPS treatment from approximately 0.5:1 to 5.5:1 in the absence of siRNAs. When splenic B cells were treated for 24 h with LPS and non-specific shRNA (t48+iNS) there was no change in the ELL2 amount or the secretory:membrane ratio relative to untreated cells. When splenic B cells were treated with LPS and shRNA to ELL2 (iell2)

the abundance of ELL2 mRNA was reduced relative to untreated or non-specific shRNA treated (iNS) and the secretory:membrane ratio was significantly reduced (Fig. 5b). Therefore, diminishing the amount of ELL2 mRNA reduced the processing of the endogenous *Igh* μ pre-mRNA to the secretory form in these cells, confirming its role in IgH expression in primary cells.

ELL2 and tandem poly(A) site selection

The ELL2 protein increases the production of secretory mRNA; this could be achieved either by enhancing the use of the promoter proximal poly(A) site or increasing exon skipping of the splice site in CH3 (γ) and CH4 (μ), or both. Some elongation factors have been shown to influence one or the other but not both reactions on non-lymphoid genes. To explicitly address mechanism(s) altered by ELL2 we separated the reaction into its two component parts. For assessing polyadenylation enhancement of ELL2, we modified a reporter plasmid driven by the *Igh* enhancer and SV40 promoter that showed plasma cell specific expression[25]. The SV40 promoter–driven plasmids were used to validate the parameters of the IgH secretory:membrane poly(A) choice in previous studies[26]. The secretory *Igh* γ poly(A) site is in competition with a downstream SV40 early poly(A) site (Fig. 6a). The *Igh* γ-derived IVS DNA between the sites is ~2 kb, versus the 3 kb between the secretory and membrane poly(A) sites in the *Igh* genes. Addition of WT ELL2 resulted in a 2.8-fold stimulation in the use of the promoter proximal poly(A) site (Fig. 6b). Addition of NH2 ELL2 showed no significant change in use of the promoter-proximal, secretory poly(A) site while the COOH ELL2 (encoding the 58 kDa form of the protein) stimulated the use of the secretory poly(A) site, consistent with its ability to facilitate CstF-64 attachment to the RNAP-II complex as shown previously (Fig. 4). The addition of shRNA for ELL2 (iell2) resulted in a decrease in the use of the secretory poly(A) site; adding back full-length ELL2 (iell2 + WT ELL2) restored secretory poly(A) site use. These results are consistent with ELL2 enhancing promoter-proximal, secretory-specific poly(A) site use.

Effect of ELL2 on splicing of alternative exons

We assayed whether the recombinant ELL2 proteins could also influence splice-site choices. Studies with the fibronectin gene containing an alternatively spliced extra domain (ED1 exon)[27–29] have shown that more efficient elongation of RNAP-II leads to more exon skipping while slowing the polymerase led to more exon inclusion. Using the ED1 alternative splicing constructs[12] as a reporter in Hep3B cells (Fig. 7a), we saw increased ED1 exon skipping with reporters driven by the *HBA1* (human α globin) promoter plus WT* ELL2 (lacking HA tag), but not *Fn1* or cytomegalovirus (CMV) promoters (Fig. 7b). This result is consistent with previous studies and confirms that ELL2 acts to enhance exon skipping with some promoters.

In A20 B cells, using the ED1 reporter driven by the *Igh* promoter and enhancer, there was an approximate 3-fold increase in exon skipping upon the addition of full-length WT ELL2 relative to empty vector (Fig. 7c). The $M_{133,138,186}I$ form of ELL2 caused an approximate 2-fold effect as did the COOH-ELL2 (58 kDa protein). The NH2 ELL2 was ineffective in changing the splicing pattern. We conclude that increased expression of the WT full-length or COOH-ELL2 facilitates significant exon skipping; this could shift the balance towards

use of the secretory poly(A) site in the *Igh* transcription unit which would otherwise be removed by the use of the 5′ splice-site encased in the last secretory exon to the M1 exon. Taking these results together with the stimulation of promoter proximal poly(A) site use and enhanced loading of CstF-64 onto RNAP-II, we conclude that the elongation factor ELL2 influences IgH pre-mRNA processing by influencing both exon skipping (splicing) and polyadenylation (model in Supplementary Fig. 4 online).

DISCUSSION

We have established the transcription elongation factor ELL2 as an essential regulator of the switch from the membrane bound to the secreted form of IgH mRNA, a fundamental step in plasma cell differentiation. ELL2 influences IgH pre-mRNA processing, shifting it away from splicing to M1 and towards use of the secretory-specific poly(A) site. The ELL2 protein accumulates primarily as a shortened form (58–59 kDa) and both the clones encoding the full-length (80 kDa) and shortened form are able to direct the binding of CstF-64 to the RNAP-II near the *Igh* promoter. Addition of the full-length ELL2 clone alters IgH processing in a B cell line while both the clones encoding the short and long forms of ELL2 drive promoter-proximal poly(A) site use and alternative splicing in model systems, recapitulating the two competing processing events occurring in the *Igh* locus.

ELL2 expression was down-modulated following transfection of plasma cells with hnRNP F, a condition we previously showed skewed IgH mRNA processing by binding to the secretory poly(A) site14. Here results from our microarray data and RT-qPCR experiments suggest that hnRNP F also suppressed ELL2 expression, most likely by binding to the multiple GRS located within the *Ell2* sequence, resulting in even further reduction of the polyadenylation versus splicing reaction. Determining the ratio of secretory to membrane specific IgH mRNA is complex and influenced at least in part by the reduction in plasma cells of several previously described *trans*-acting factors, U1A, hnRNP F and some serine-arginine rich (SR) proteins30,31,14. Over-expression of the polyadenylation-cleavage factor CstF-64 has been shown to influence the IgH RNA processing choice in chicken DT40 cells32. While transfection of plasma cells with hnRNP F decreased ELL2 expression and Ig secretion, it did not influence the abundance of CstF-64, U1A or the SR proteins (see microarray data).

We incorporate our recent findings into a model of the current knowledge of IgH mRNA processing. ELL2 plays a key role by directing association of the polyadenylation factor CstF-64 to RNAP- II in plasma cells. ChIP experiments conducted with plasma cells show the polyadenylation factors and ELL2 were associated with the *Igh* gene more efficiently than in B cells, likely due to the increased phosphorylation of the CTD of RNAP-II3. Here we show by inhibiting ELL2 expression by siRNA that the binding of the polyadenylation factor CstF-64 to RNAP-II depended on ELL2. As a consequence of loading onto RNAP-II, the polyadenylation factors were present at a high local concentration and acted on the weak secretory-specific poly(A) site to direct its recognition and cleavage. We hypothesize that ELL2 prevents the recognition of the weak alternative splice site in the CH3 exon (γ or CH4 in μ), used to produce the membrane-specific form of Ig heavy chain. Indeed, factors that speed RNAP-II elongation rate are known to aid exon skipping12. Since the splice site and

poly(A) site usage are in direct competition33, the secretory-specific poly(A) site is acted on by the polyadenylation factors loaded on RNAP-II when ELL2 abundance is higher. The balance tips towards promoter-proximal polyadenylation and thus to Ig secretion. Promoter-proximal poly(A) site use is stimulated by at least one other elongation factor, Cdc73 also known as parafibromin, constitutive in non-lymphoid cells13. ELL2 is unique among transcription elongation factors thus far described in influencing exon skipping, enhancing promoter proximal poly(A) site choice and demonstrating developmental regulation.

CstF-64 was associated with the *Igh* μ and γ gene segments along their entire lengths, indicating that the polyadenylation complex remains bound to the RNAP-II even as it processes the mRNA and beyond. The polyadenylation factors may remain tightly associated with the polymerase so as to act at the downstream membrane poly(A) site should the first poly(A) site fail to cause processing. This linkage may occur because different subunits of the polyadenylation complex are involved in the RNA binding versus polymerase association. For example, CstF-64 binds GU-regions in RNA but is found in a trimer complex with CstF-50 and CstF-77; but it is CstF-50 that binds to RNAP-II34, 35. Additionally, the CstF trimer is found associated with a number of other poly(A) site processing factors in a large complex of 85 different proteins. Neither ELL1 nor ELL2 were found in this large preformed complex35, so if ELL2 and CstF-64 associate directly it is only transient while their major interaction is clearly through the phosphorylated CTD of RNAP-II, based on our previous studies 3. It is also clear that at least 400 nucleotides of RNA has to extrude from mammalian RNAP-II before processing takes place36. In yeast, at least for some genes, the polyadenylation factors add onto the polymerase as it transits close to the 3′ end. In human cells there is growing evidence that the polyadenylation factors may add to the RNAP-II complex much nearer the promoter in some but not all genes13, 37, 38. Our observation that Ser-2 phosphorylation of the CTD of RNAP-II occurs at or near the promoter in the *Igh* γ gene segment as opposed to near the 3′ end, as seen in yeast genes3, indicates fundamental differences from yeast that facilitate association of ELL2 and CstF-64 with RNAP-II.

Promoters and elements within genes are known to direct alternative splicing; it has been suggested that they might do this by directing the association of specific elongation and RNA processing factor combinations and concentrations specific to the gene 39. We show that ELL2 strongly binds and influences the *Igh* promoter but other promoters are influenced as well; the SV40 driven reporter plasmid showed more proximal poly(A) site choice with addition of ELL2 while exon skipping was enhanced by ELL2 with the *Igh* and *HBA1* promoter driven reporters, but not the CMV promoter. There is also an association of ELL2 and CstF-64 with housekeeping genes like *Actb* and *Gapdh*. Thus ELL2 may have more generalized effects beyond Ig secretion and regulate other genes; exploring other targets of ELL2 should prove informative for plasma cell biology.

METHODS

Mouse cells

B cell lines were described previously14. Resting splenocytes, $3–6 \times 10^7$ cells were pan-T depleted with CD90 microbeads and AutoMACs (Miltenyi Biotec). The remaining cells at

$4-5\times10^6$ cells/ ml were cultured in cRPMI plus 20 μg/ ml LPS (*Escherichia coli* 0111:B4) for 72 h. Transfections were performed with the Amaxa mouse B cell nucleofector® kit according the manufacturer's protocol. Animal experiments were reviewed and approved (assurance number A3187-01) by the Institutional Animal Care and Use Committee at the University of Pittsburgh.

Microarray data

Biotin-labeled cRNAs were synthesized from poly(A) RNA samples and hybridized with Affymetrix U74Av2 murine oligonucleotide chips. Each cRNA sample was hybridized separately and each data comparison was a result of two separate hybridizations. After normalization of signals among different chips, each probe was scored either "Present", "Absent" or "Marginal" by the Affymetrix software depending on the overall signal strength. Significance Analysis of Microarray (SAM) was performed as described[40]; the induction and repression cutoff point was taken as two when seen in both samples.

Antibody preparation

The antibody R4502 against ELL2 was raised to the epitope GCLMNKKARISHLTNRV as previously described [3]. Antibody R4213 to ELL2 was prepared (Rockland, Inc.) to CSHKKSKKKSKKKHKEKDQIKK after coupling through the terminal Cysteine to KLH. Both were affinity purified over a sulfo-link column (Pierce, Rockford. IL) coupled with the immunizing peptide. Specificity of R4213 for ELL2 is shown in Supplementary Figure 1.

RT-qPCR and qPCR

RNA was isolated with the Ultraspec™ reagent (Biotex), treated with DNA-free™ (Ambion), and reverse transcribed with dT and Superscript II RT (Invitrogen). Quantitative PCR (qPCR) was performed with SYBR Green 2x PCR Master Mix (Applied Biosystems) and 100 nM primers, on an Applied Biosystems 7900HT. Primers were purchased from Integrated DNA Technologies; the sequences are listed in Supplementary Table 1 or previously published[3]. The qPCR program used for DNA samples was: Taq activation for 12 min at 95 °C, followed by 40 cycles of a 15 sec denaturing step at 95°C and a 1 min annealing step at 60 °C.

Statistics

Data were analyzed with Graphpad software PRISM™ using the one-way ANOVA with the Tukey's pairwise post test to compare the values with added factors (ELL2 etc) vs the empty vector in figure 4a, figure 6a, figure 7b and figure 8c. The Mann-Whitney-Wilcoxon test was used for several samples in Fig. 5a. The error bars in all the figures represent the standard error of the mean (S.E.M.).

Chromatin immunoprecipitaion assay and real time PCR

Sheared chromatin was prepared and immunoprecipitated as described[3,41], using the magnetic bead ChIP-IT™ express kit from Active Motif. DNA was prepared using directions supplied by the ChIP-IT kit (handbook 102026) except that the de-crosslinking step was conducted at 65°C overnight, the proteinase K digestion was increased to 2 μl of 10

mg/ml proteinase K at 37°C for 2 h and the DNA isolated on QIAquick® PCR purification kit (Qiagen). The *Igh* μ probes are listed in Supplementary Table 1. The *Actb* promoter3 with RNAP-II was always run as a control for normalization3.

Transfections for site choice in IgH and tandem poly(A) reporters

Cells were transfected with Lipofectamine 2000 (Invitrogen) per manufacturer's instructions. The IgG2b heavy chain reporter illustrated in Figure 1a was described previously19. The tandem poly(A) reporter was derived from a pSV2gpt containing the Ig heavy chain enhancer, previously described25, to which was added a 2 kb *Sst* I to *Sma* I fragment from the IgG2b IVS between the sec poly(A) site and M1, upstream of the SV40 early poly(A) site.

Image clone #4822236 (ATCC) for human ELL2 in Bluescript was digested with *Hind* III and *Sma* I, filled in, then cloned into the pEF4B (Invitrogen) eukaryotic expression vector (See Supplementary Methods). To make COOH ELL2, an *Alw* NI fragment was blunt ended and cloned into pEF4/HisC (Invitrogen). All pEF4B clones encode an amino-terminal Express Tag™ to facilitate nuclear localization and distinguish the product from the endogenous protein.

The expression plasmid for PC4 in CMVsport6 was obtained from ATCC (MGC-13711). The human CstF-64 expression vector is driven by the pEF1/mycHisB promoter as characterized previously 42. The shortened form of CstF-64 (dominant negative) which lacks the last 282 base pairs of cDNA but contains the RNA binding and CstF-77 interaction domains is a *Sand* I restriction enzyme truncation of CstF-64 with an HA tag added (5′-GTCCAGGCGCCTACCCATACGACGTCCCAGACTACGCTGCTAGT-3′ and 5′-CTAGACTAGCAGCGTAGTCTGGGACGTCGTATGGGTAGGCGCCTG-3′). Each of these expression clones produce full length proteins capable of being recognized by the appropriate antibodies (data not shown). RNA was harvested after 48 h. The ratios of secretory to membrane or proximal to distal site use in the mRNAs were obtained using RT-qPCR with the IgG2b or tandem poly(A) primers listed in Supplementary Table 1.

shRNA studies

Oligonucleotides for hairpin siRNAs were inserted in the pSilencer 2.1-U6 hygro expression vector (Ambion). These plasmids and the IgG2b heavy chain reporter (1ug each) were co-transfected into A20 or J558L cells with Lipofectamine 2000. The sequence of the 3′ UTR of ELL2 mRNA contained in the shRNA (#482) most effective on ELL2 was: 5′-CCCAGTAGCTACACTGCTTCC-3′. The non-specific (NS) shRNA construct was purchased from Ambion. The same shRNAs were used throughout.

Exon skipping/ inclusion assay

Hep3b cells were obtained from ATCC (#HB-8064) and cultured as directed. Two RT-PCR products are produced from the ED1 reporter mRNA; the shorter (230 bp) corresponds to the mRNA with the skipped product while the larger product (500 bp) corresponds to RNAs which include the alternative ED1 exon 43. The ratio of the two products from Hep3B cells

transfected with the reporter was determined by digitally imaging ethidium bromide stained agarose gels on a Kodak Image Station 2000R.

For transfection in A20 cells, the *HBA1* promoter driven splicing reporter was replaced with the *Igh* promoter by digesting and blunting a *Bss* HI site then digesting with *Eco* RI and inserting the 441 bp *Hind* III (blunted) and *Eco* RI fragment of the *Igh* promoter construct, pJ558PR (CW). To this construct was added the 3 kb *Eco* RI fragment containing Ig enhancer from MPC-11 (ref. 43). Transfections were performed as described above except that 2 ug of DNA, a 1:1 reporter to factor ratio, was used. The ratio of the skipped to included products were determined by RT-qPCR using the primers listed in Supplementary Table 1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

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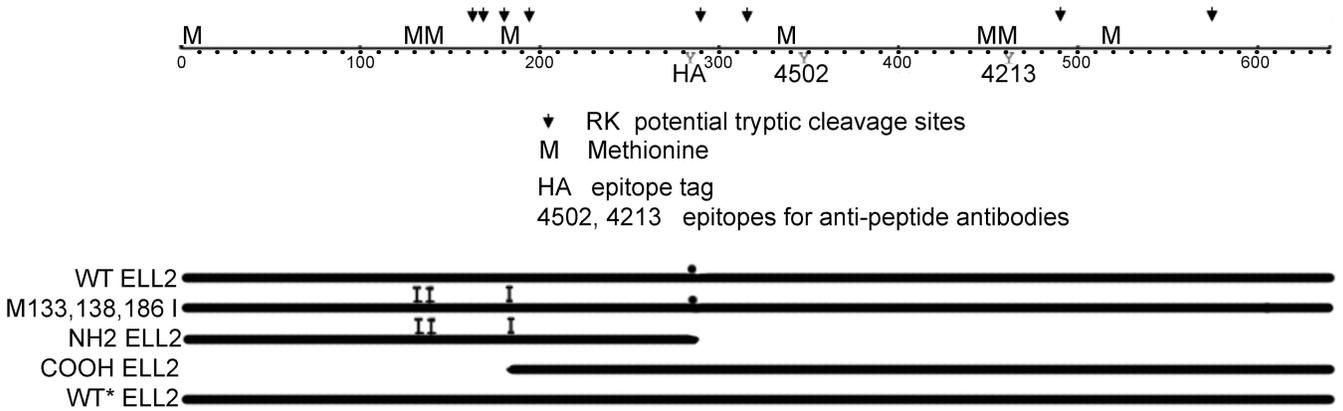a *Igh* gene and mRNA products
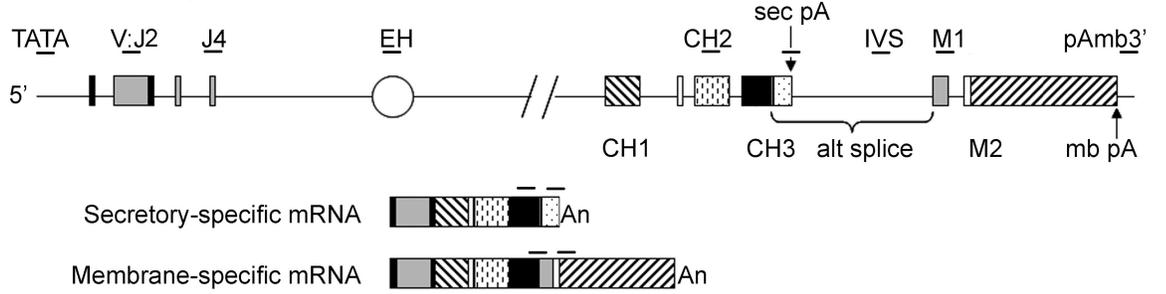
b ELL2 schematic and clones

Figure 1. Schematic of the Ig gamma 2b reporter gene

(a) The positions of secretory and membrane pA sites and the CH3 to M1 splice are indicated. The resulting mRNAs are depicted below the gene. TATA represents the location of the *IgH* promoter used in ChIP experiments. Bars above *IgH* gene and mRNA indicate location of primer pairs for Q-PCR, see Supplementary Table 1 for sequence of primers. **(b)** Schematic of the ELL2 protein and clones used. Indicated are the positions of the in-frame methiones (M), the position of the inserted hemaglutinnin (HA or ●) tag, the location of the peptide epitopes for the rabbit polyclonal antibodies (R4502 and R4213) described here, and potential RK tryptic cleavage sites (arrows). Conservation of these sequences in vertebrates is indicated in Supplementary Table 2. WT ELL2, full length with hematagglutinin (HA) tag (●); $M_{133,138,186}$I ELL2 showing methionine to isoleucine substitutions; NH2 ELL2, a.a. 1–285; COOH ELL2, corresponding to a 58 kDa ELL2 protein with start site near M_{186}; and WT* ELL2, lacking the HA tag.
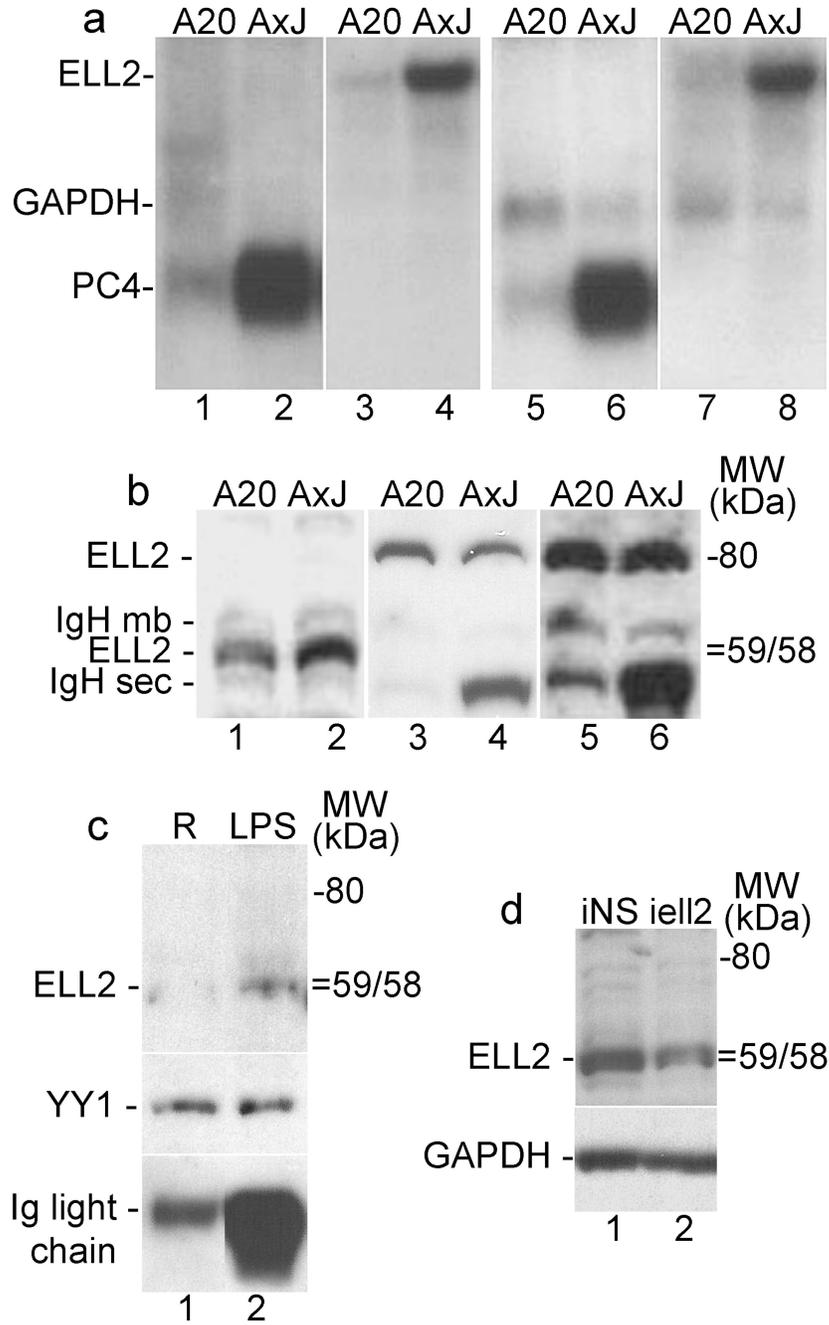
Figure 2. ELL2 mRNA and protein are increased in cells stimulated to secretion

(a) Hybridization of polyA$^+$ RNA from the mouse B cell line (A20) lanes 1, 3, 5, 7 and the plasma cell (AxJ) lanes 2, 4, 6, 8. Filters probed with (^{32}P)-U labeled complementary RNA for PC4 (lanes 1, 2) or ELL2 (lanes 3, 4). Duplicate samples run on same gel which was cut and aligned after hybridization. The blots were reprobed with complementary RNA for GAPDH (lanes 5–8). ELL2 mRNA 4 kb in size by comparison with 28 S rRNA (4 kb), GAPDH (1 kb), and PC4 mRNA (0.68 kb). (b) Immunoblot of whole cell lysates from A20 and AxJ cell lines probed with R4213 polyclonal antibody to ELL2 (lanes 1, 2). Same blot

was stripped and reprobed with 83104 monoclonal antibody to ELL2 (lanes 3, 4). Darker exposure of same blot (lanes 5, 6) revealed the membrane form of IgG heavy chain, the loading control. **(c)** Immunoblot of Triton X-100 lysates of splenic B cells, resting (R, lane1) or stimulated (LPS lane 2) probed with antibodies including R4213 for ELL2, SC-1703 for YY1, a transcription factor shown not to change between B cell stages, and anti-mouse IgG for Ig light chain. **(d)** Immunoblot of Triton lysates of mouse 3T3 cells transfected with nonspecific shRNA, iNS, (lane 1) or ELL2 interfering shRNA, (iell2, lane 2) probed with R4213, reprobed with GAPDH.
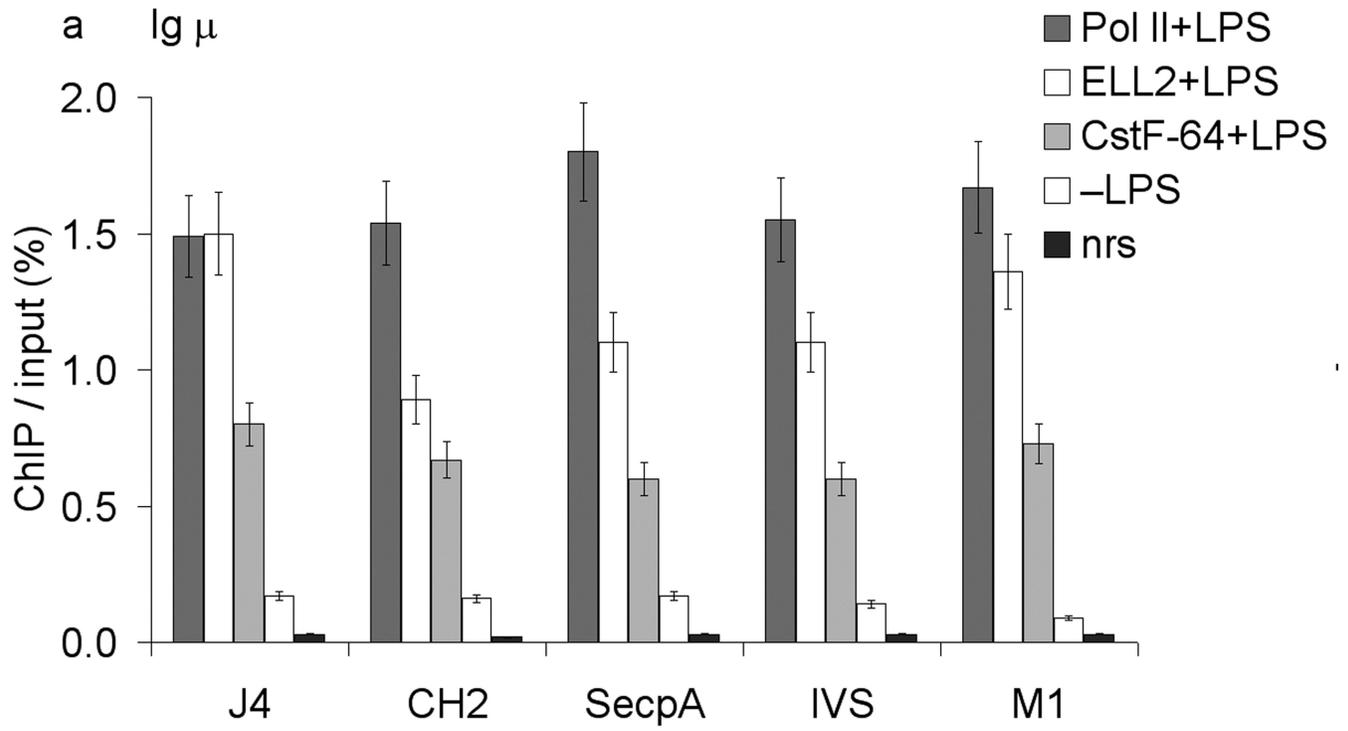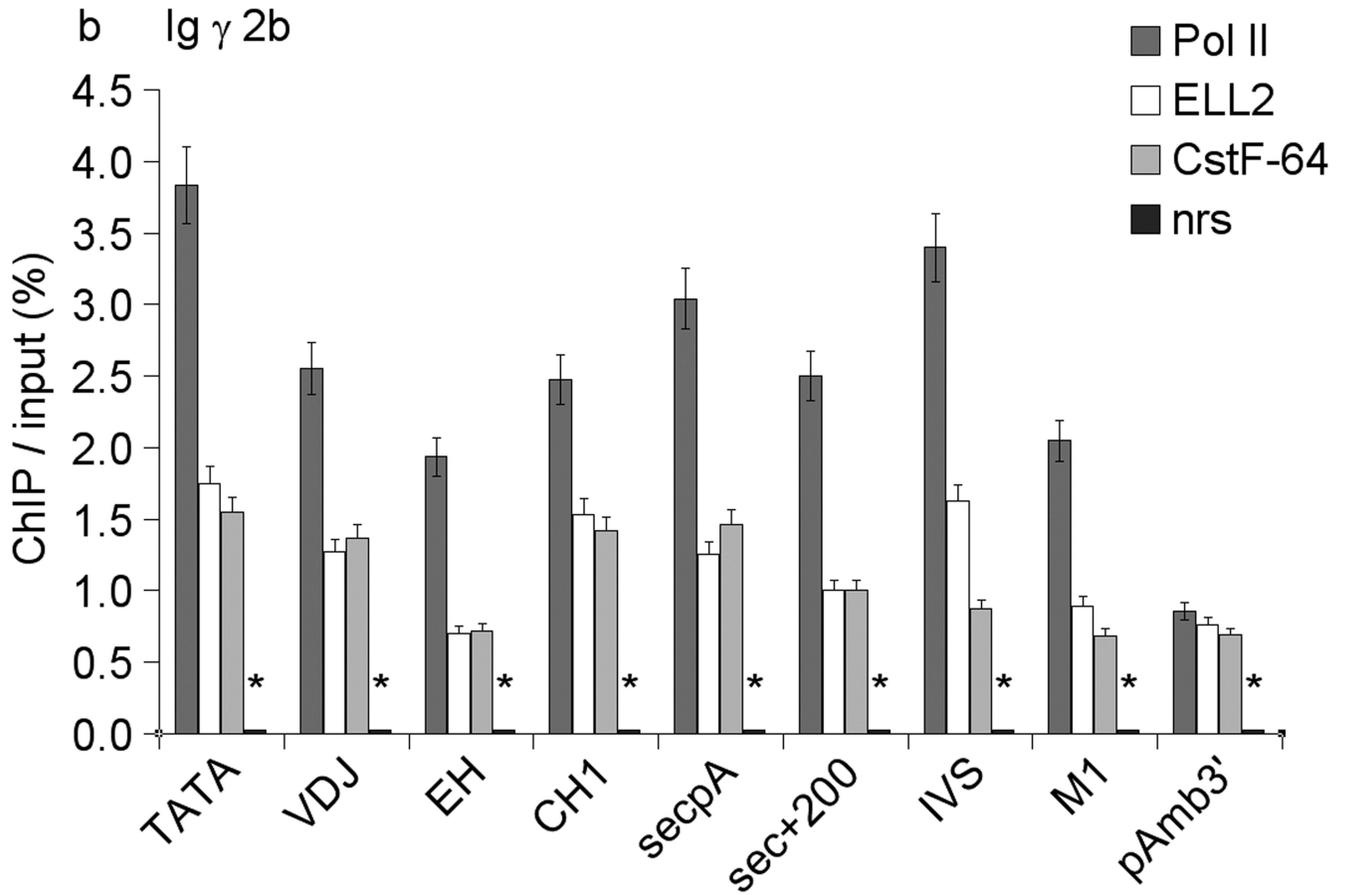
Figure 3. ELL2 and CstF-64 cotrack with RNA Polymerase II across the *Igh* gene

Chromatin immunoprecipitations performed with antibodies to RNAP-II (pol II), ELL2, CstF-64, or normal rabbit serum (nrs). **Panel a**. Primary mouse splenic B cells harvested immediately (–LPS) or treated for 72 hours with Lipopolysaccharide (+LPS) to induce Ig secretion; Pol II+LPS, ELL2+LPS and CstF-64+LPS, nrs +LPS. The minus LPS values for all three antibodies have been averaged. Probes to the mouse *Igh* mu gene were used for qPCR, from J4 to M1, see Supplementary Table 1. **Panel b**. J558L plasma cells were transfected with the IgG2b heavy chain gene. The location of probes used are diagrammed in Figure 1 a. Error bars in Panels a and b represent the standard error S.E.M.; *n* = from 3 to 6; significance (P < 0.05) calculated by ANOVA and indicated by *.
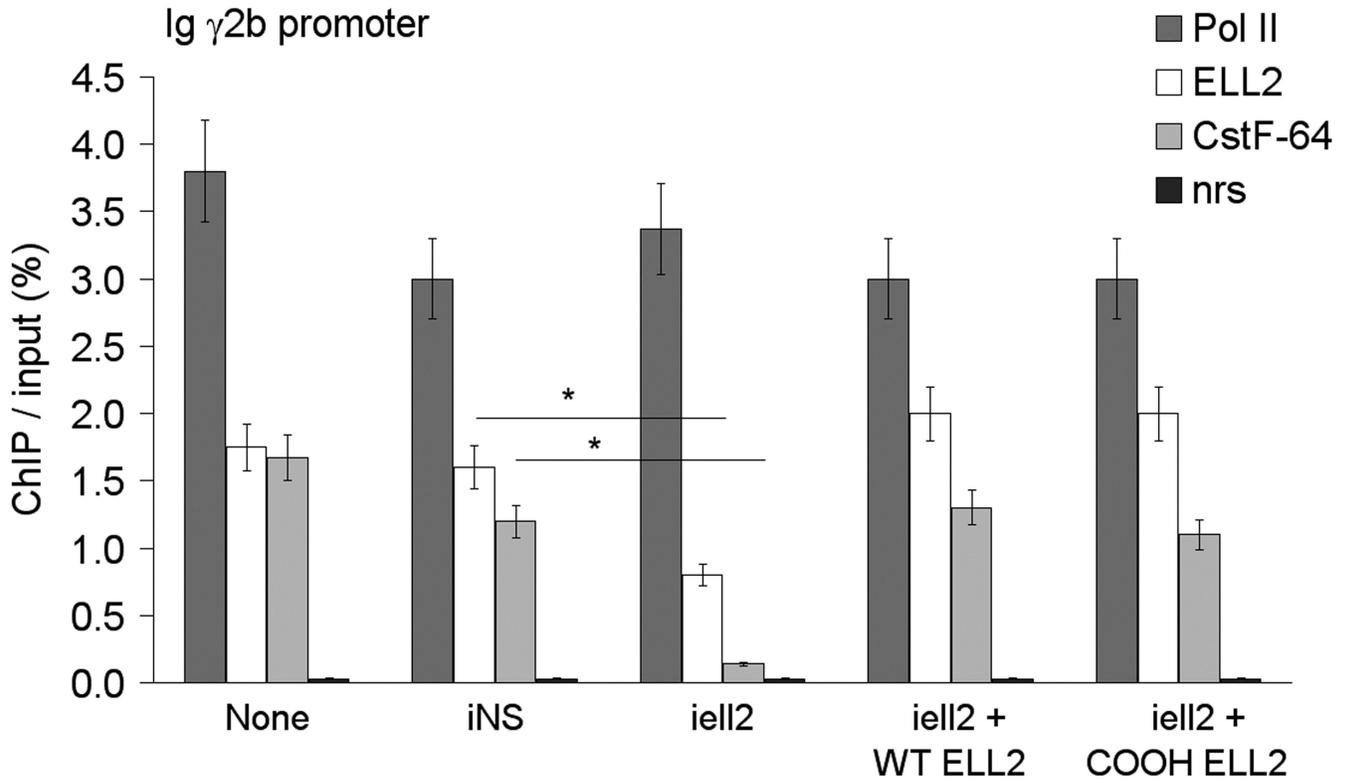
Figure 4. The siRNA to ELL2 interferes with Chromatin immunoprecipitation of CstF-64 at the *Igh* promoter region

The J558L plasma cells were transfected with the IgG2b reporter plasmid with intact Ig promoter and enhancer, see Figure 1a, and either left untreated (none), or incubated for 24 hours with: the nonspecific shRNA (iNS); the anti-ELL2 shRNA (iell2); iELL2 plus intact WT ELL2 clone (iell2 + WT ELL2); or iell2 plus the ELL2 clone starting at M_{186} (iell2+ COOH ELL2). Chromatin IP was performed with antibodies against RNA Polymerase II (Pol II), ELL2, CstF-64, or normal rabbit serum (nrs). The resulting sheared DNA was uncrosslinked and amplified in a real-time qPCR. Primers for the 5' end of the IgG2b gene (TATA) were used for all samples. $P = <0.01$ indicated by *. Error bars in represent the S.E.M.; $n = 3$ to 6.
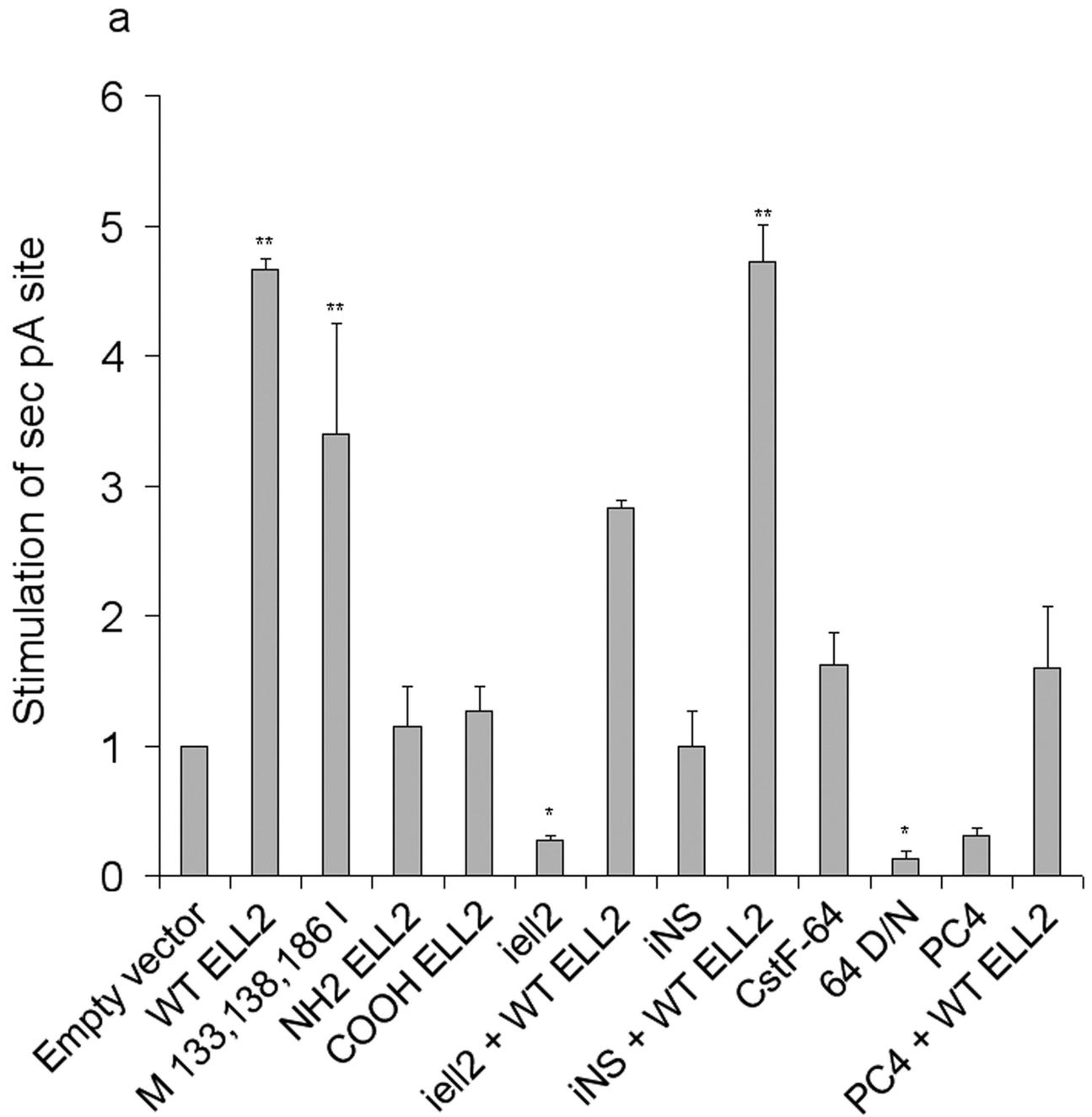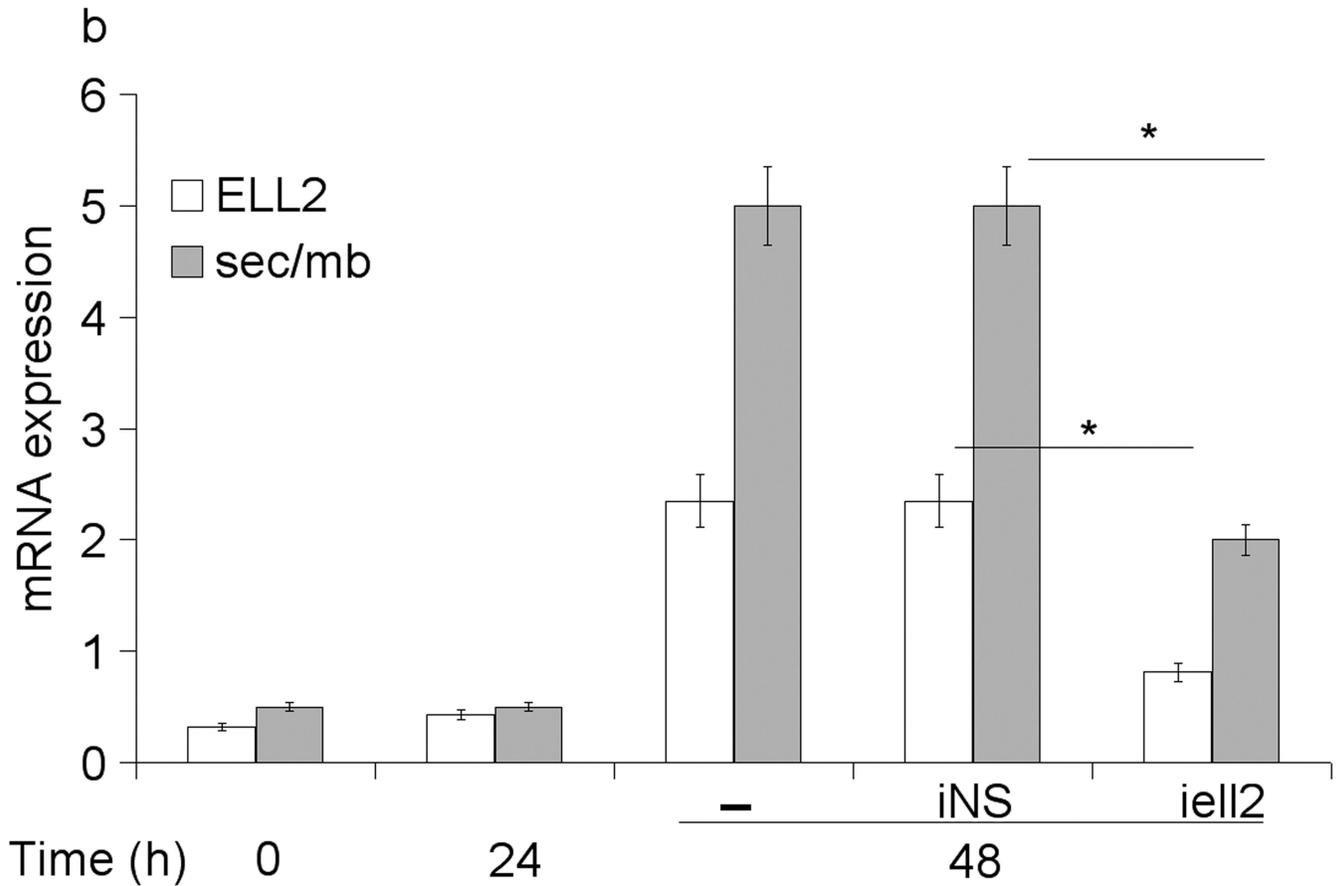
Figure 5. ELL2 enhances the production of secretory mRNA
Production of secretory-specific or membrane-specific RNA was assessed by RT-qPCR using primers indicated in Figure 1a. and Supplementary Table 1. **Panel a**. ELL2 clones (Fig 1b) were co-transfected with the *Igh* γ2b reporter. The secretory to membrane mRNA ratios were determined and normalized to the empty vector which was set as 1. The iell2 is the interfering shRNA cotransfected with the reporter. iNS is a nonspecific shRNA sequence. The CstF-64 dominant negative mutant is abbreviated 64 D/N. PC4 is another co-transcription factor. **Panel b**. Primary mouse splenic B cells were either isolated (time 0), cultured with LPS (24 h), or treated with iNS, iell2 or nothing (−) and incubated an additional 24 hours. The amount of ELL2 mRNA (vs HPRT) or the ratio of secretory- to membrane-specific endogenous IgM mRNA was determined. Error bars represent S.E.M.; *n* = 3. Samples were analyzed by ANOVA with Tukey's post tests and significance ($P < 0.01$) indicated with **. A comparison of iell2 vs empty vector and 64 D/N vs empty vector displayed significance ($P = <0.05$) using a pairwise Mann-Whitney-Wilcoxon test, as indicated by *.
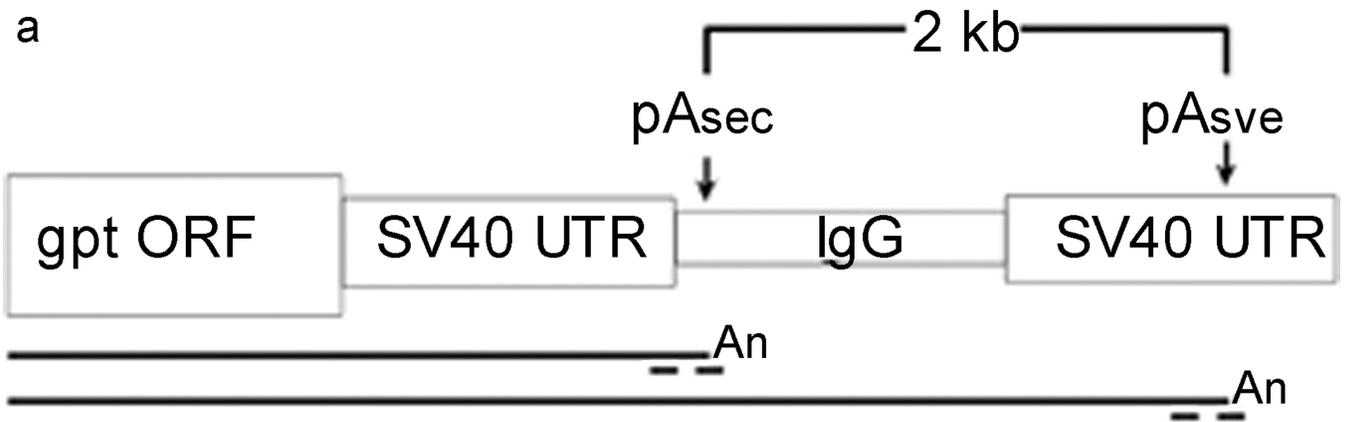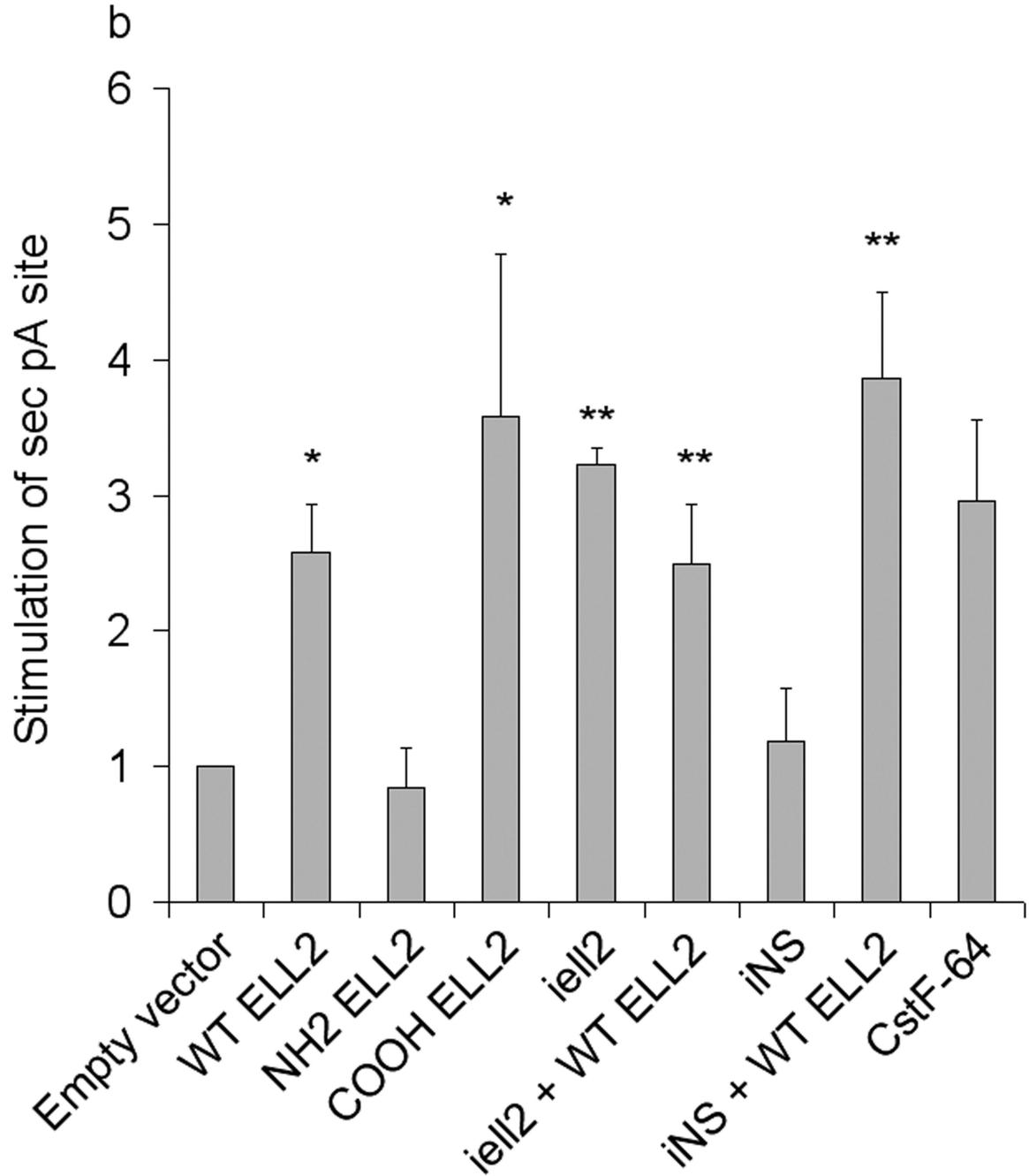
a

Figure 6. ELL2 influences poly(A) site choice

Use of the proximal *Igh* secretory (pA$_{sec}$) versus distal (pA$_{SVE}$) poly(A) sites was assessed by RT-qPCR of the mRNA products. **Panel a**. The strategy for assessing poly(A) site use is diagrammed. Heavy lines below the map indicate the two messages produced from the poly(A) reporter. Bars below indicate the locations of qPCR primers; primer sequences listed in Supplementary Table 1. **Panel b**. A20 B cells were transfected with the poly(A) site reporter and plasmids for the indicated factors, see Figure 1c for the ELL2 constructs. Ratio of proximal to distal poly(A) site usage are compared to cells transfected with empty vector,

which was set to 1. The values obtained with the addition of the various factors were compared to the control via ANOVA with a Tukey's post test. Values of $P < 0.01$ and $P < 0.05$ are indicated in the graph by ** and * respectively. Error bars are indicate S.E.M. and $n = 3$ to 6.
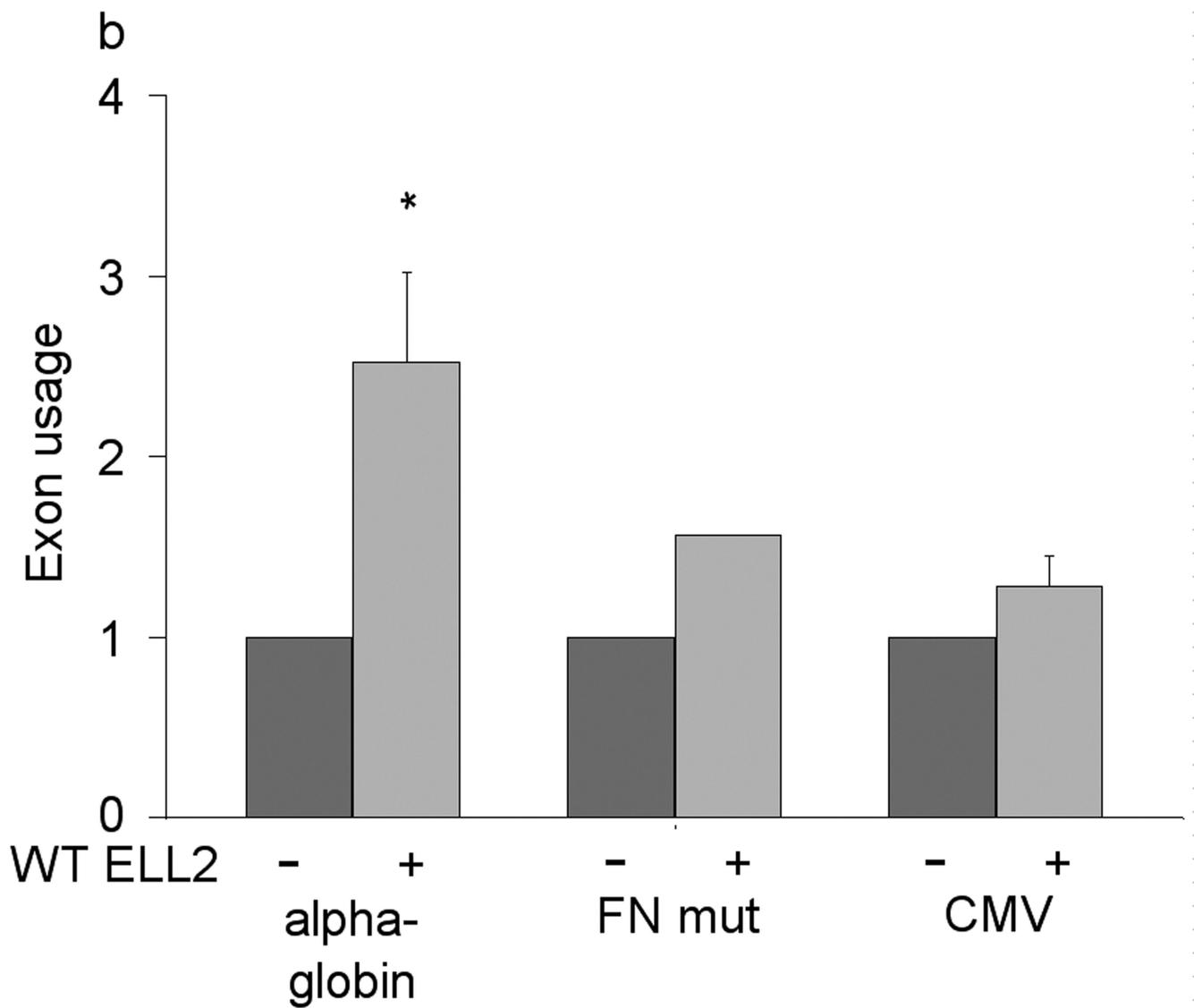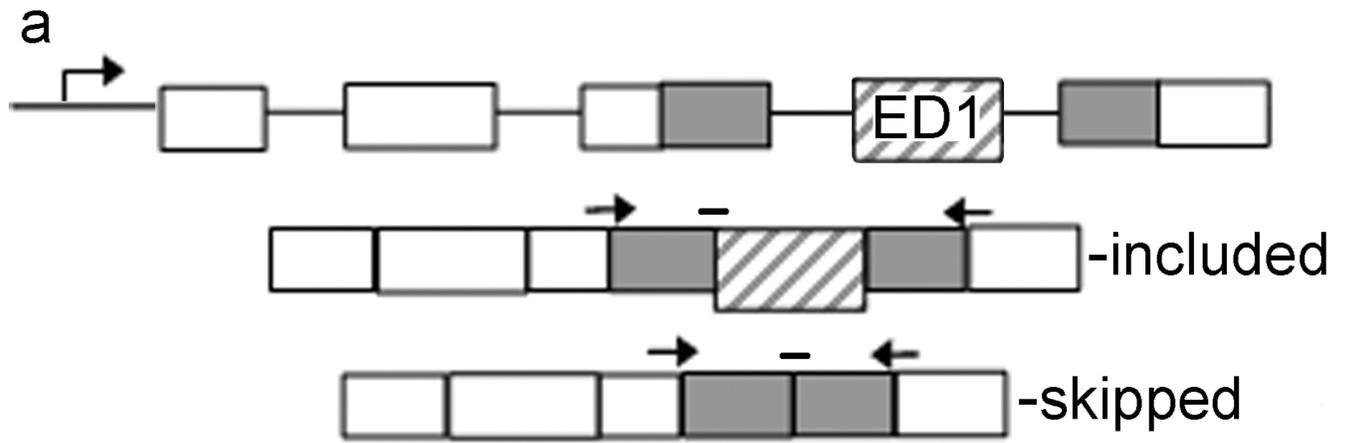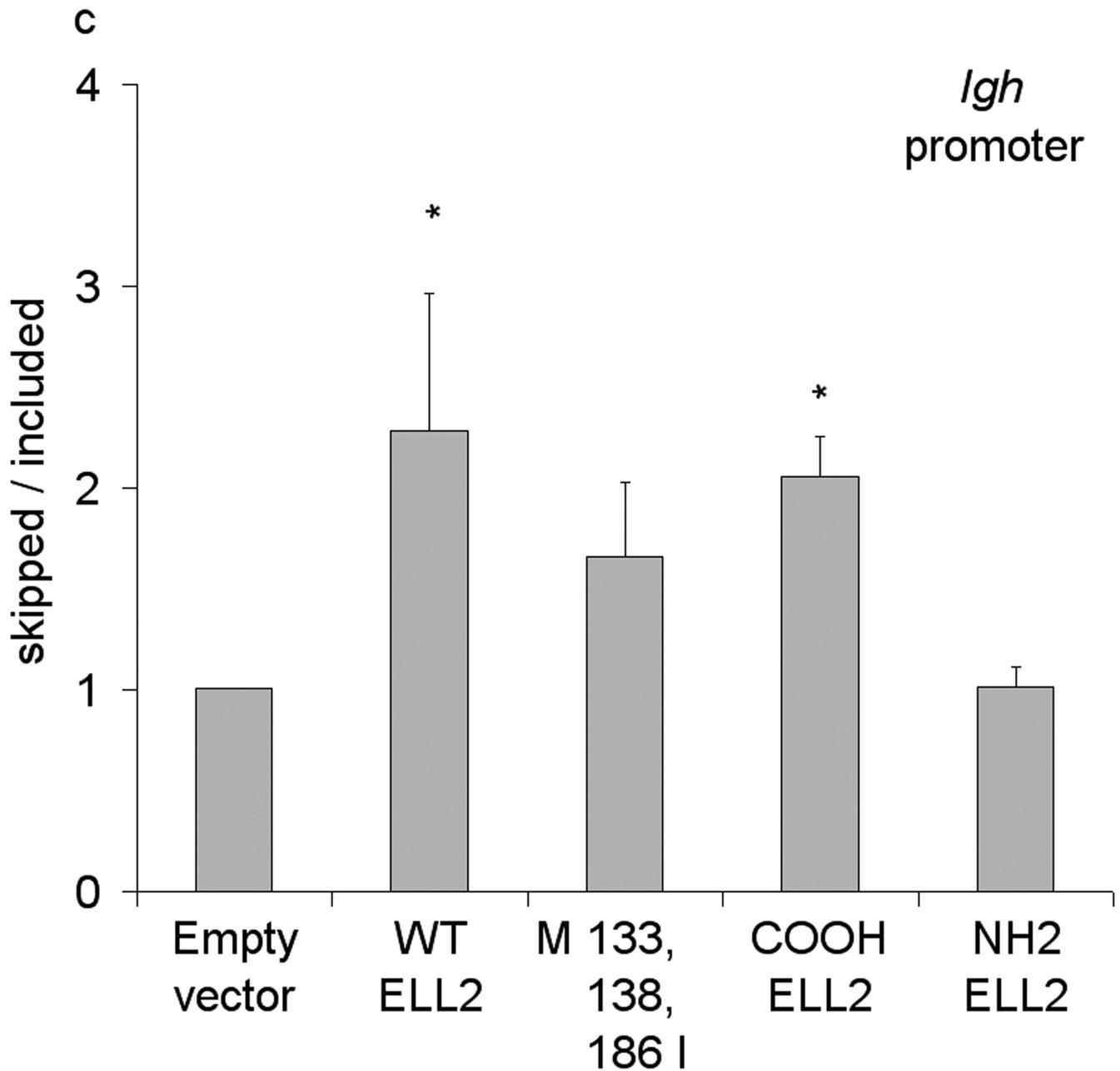
Figure 7. ELL2 enhances exon skipping

Panel a. Diagram of the splicing reporter with included ED1 exon or skipped products indicated. Large arrow indicates promoter; small arrows, the position of the PCR primers. Bars indicate 3' primers paired to the 5' PCR primer for RT-qPCR. **Panel b**. Reporters containing the indicated promoters were co-transfected with or without WT* ELL2 into Hep3B cells. Inclusion or skipping of the ED1 exon was assessed by low cycle PCR using the primers described in Methods. Skipped versus included ratios are compared to cells co-transfected with empty vector, set to 1. **Panel c**. *Igh* promoter and enhancer drive the ED1 splicing reporter; A20 B cells were co-transfected with the ELL2 clones and the splicing

reporter. The mRNA expression was assessed by RT-qPCR using primers in Supplementary Table 1. Ratio of skipped to included products are compared as in Panel b. Error bars represent S.E.M.; n = 3 to 6. Values obtained with ELL2 clones were compared to the control via ANOVA with a Tukey's post test and $P < 0.05$ indicated in the graph by *.

Table 1

mRNA expression.

	Elongation factor ELL2	Integrin alpha 4	Retinoblastoma-like (p107)
A20/AxJ[*]	**0.19**	**0.23**	**0.45**
F+AxJ/AxJ[*]	**0.33**	**0.29**	**0.45**
A20 B cell[**]	0.40	0.03	0.11
AxJ Plasma cell[**]	2.12	0.09	0.26
F+AxJ[**]	0.66	0.02	0.14
A20/AxJ[**]	**0.19**	**0.32**	**0.41**
F+AxJ/AxJ[**]	**0.31**	**0.25**	**0.55**

[*] Affimetrix array data showing the selected genes whose expression was changed by more than 2-fold in cell to cell comparisons with F+AxJ versus AxJ and AxJ versus A20. Probe sets:103892_r_at (ELL2); 102655_at (integrin alpha 4); 104476_at (Rb p107). Average of three separate determinations with S.E.M.s less than 5 %.

[**] Values are $2^{-\Delta Ct}$ comparisons relative to HPRT mRNA. Primers for RT-qPCR are listed in Supplemenatry Table 1. Average of 6 real-time qPCR determinations with 2 independent cell samples; S.E.M. less than 5 %.