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ALTERATION OF SC35 LOCALIZATION BY TRANSFECTION REAGENTS

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Abbreviations: NS, Normal SC35; ESS, Enlarged SC35 Speckles; CG, Cytoplasmic Granules.

Abstract

Transfection is a powerful tool that enables introducing foreign nucleic acids into living cells in order to study the function of a gene product. Ever since the discovery of transfection many side effects or artifacts caused by transfection reagents have been reported. In this report, we show that the transfection reagent, JetPRIME alters the localization of the splicing protein SC35 widely used as a nuclear speckle marker. We demonstrate that transfection of plasmids with JetPRIME leads to enlarged SC35 speckles and SC35 cytoplasmic granules. By contrast, transfection of the same plasmid with Lipofectamine 3000 does not have any effect on SC35 localization. The formation of SC35 cytoplasmic granules by JetPRIME-mediated transfection is independent of exogenous expression by plasmid and although similar in morphology they are distinct from P-bodies and stress granules. This method of transfection affected only SC35 and phosphorylated SR proteins but not the nuclear speckles. The JetPRIME-mediated transfection also showed compromised transcription in cells with enlarged SC35 speckles. Our work indicates that the use of JetPRIME alters SC35 localization and can affect gene expression and alternative splicing. Therefore, caution should be exercised when interpreting results after the use of a transient transfection system, particularly when the subject of the study is the function of a protein in the control of gene expression or mRNA splicing.

Keywords: Transfection, JetPRIME, Splicing, SC35, Speckles, Cytoplasmic granules

1. Introduction

Transfection is a method of delivering foreign nucleic acids (DNA and RNA) to the living cells. However, the term "transfection" was initially used to represent the procedure of introducing the λ DNA directly to *E.coli* [1,2]. Ever since the usage of DEAE-dextran to transfer the nucleic acids to mammalian cells [3], many transfection reagents have been developed and commercialized. Transfection methods and transfection reagents have evolved since its outset, from transfection of cell lines to transfection of organs/tissues. Today there are personalized transfection reagents to deliver nucleic acids or proteins to cell lines that differ based on their culturing condition (adherent or suspension), cell type and the level of ease of transfection. There are transfection reagents developed even for *in vivo* delivery to specific organs like liver by exploiting the membrane receptors that these organs carry (like in vivo-jetPEI®-Gal reagent- Polyplus-transfection S.A, Illkirch, France).

Depending on the duration that the transfected nucleic acid exists in the cell and the integration status of nucleic acids into the cell's genome, transfection is subclassified into two types namely transient or stable. In transient transfection, the nucleic acid exists for a limited duration and does not integrate into the genome whereas, in stable transfection, the nucleic acid is integrated into the cell's genome and hence transferred generation after generation and thus persists in the cell for a longer duration [4]. The choice between transient or stable transfection depends on the nature of the question to be addressed by considering the time required, the degree of expression, the toxicity and above all the side effects.

There are three main methods of transfection: chemical, biological and physical methods. The most widely used chemical methods include the use of carrier reagents such as calcium phosphate, cationic polymers and cationic lipids. These cationic carriers form positively charged complexes along with negatively charged nucleic acid thus enabling the attraction of this complex to the negatively charged cell membrane. Later the carrier reagent-nucleic acid complex passes through the cell membrane. The biological method involves the virus-mediated transfection and is otherwise

called transduction. This method integrates the transgene into host genome (stable transfection) and is widely used in clinical research for *in vivo* studies. The physical method involves the direct introduction of the nucleic acids to the cell's cytoplasm or nucleus. The physical methods are electroporation, biolistic particle delivery (particle bombardment), direct microinjection and laser-mediated transfection (phototransfection). The pros and cons of using each method of transfection are described elsewhere [5].

In recent years, several researchers reported the side effects or artifacts caused by various methods of transfection. These off-target effects are at the epigenetic, metabolomic, transcriptomic and proteomic levels [6–8]. All these side effects might often be misinterpreted as the function of the product expressed by the transfected gene. In this study, we report an artifact created by the transfection reagent JetPRIME when used for transfecting a plasmid DNA, which alters the localization of the splicing protein SC35, commonly used as a nuclear speckle marker.

2. Results

2.1. Transfection using JetPRIME affects the localization of SC35

Several off-target effects such as alteration of gene expression and protein levels are attributed to the reagents used for transfection [6–8]. During a transient transfection experiment using JetPRIME, we serendipitously observed that the localization of the splicing protein SC35 was altered. SC35 staining is usually used as a nuclear speckle marker. To further investigate this observation, we transiently transfected pEGFP-C1 vector in U2OS cells using JetPRIME and stained the cells for SC35. We observed three different phenotypes. Firstly, some cells contained bigger and rounded SC35 speckles that we called enlarged SC35 speckles (ESS) instead of its usual speckled pattern as observed in the nucleus of untreated cells (**Figure 1A, 1B and 1E**). Secondly, some cells showed SC35 foci in the cytoplasm, we called these SC35 foci as cytoplasmic granules (CG) (**Figure 1B and 1E**). Thirdly, some cells showed enlarged SC35 speckles (ESS) along with cytoplasmic granules (CG) (**Figure 1B and 1E**). This result suggests that the use of JetPRIME to transfect plasmids could be the prime cause for the observed alteration of the SC35 localization.

We further investigated whether transfection of the same pEGFP-C1 plasmid in the same U2OS cells using another transfection reagent, Lipofectamine 3000, would also affect the localization of SC35. In spite of similar transfection efficiency between JetPRIME and Lipofectamine 3000 (**Figure 1F**), transient transfection with Lipofectamine 3000 reagent did not alter SC35 localization as previously seen with JetPRIME (**Figure 1C, 1D and 1G**). The number of cells with the altered SC35 localization due to transfection using JetPRIME very well correlates with the number of GFP positive cells (**Figure 1F and 1G**). This strongly suggests that the change in the localization of SC35 is specific to transfection using JetPRIME and not Lipofectamine 3000.

Since SC35 is the widely used marker of nuclear speckles and the nuclear speckles are dynamic structures that differ depending on the cell type [9], we investigated whether the effect of JetPRIME-mediated transfection on SC35 localization could also be observed in a different cell line.

We selected HeLa cells and transfected the same plasmid pEGFP-C1. After transfection with JetPRIME, very similar to U2OS cells, HeLa cells also showed cells with enlarged SC35 speckles (ESS) and cells with cytoplasmic granules and enlarged SC35 speckles (CG and ESS) (**Supplementary Figure 1B and Supplementary Figure 1E**). However, cells with only CGs were not observed in HeLa cells as seen in the case of U2OS cells on using JetPRIME as transfection reagent. Since this could be attributed to higher transfection efficiency (GFP positive cells) in HeLa cells than in U2OS cells by JetPRIME (**Figure 1F and 1H**), we tested whether increasing the transfection efficiency in U2OS cells by transfecting high amount of plasmid could eliminate the cells with only CGs. However, this was not the case, cells with only CGs (**Supplementary Figure 1F**) were still observed suggesting there could be slight differences in the SC35 localization depending on the cell type. The efficiency of transfection estimated by the number of GFP positive cells was higher with JetPRIME than with Lipofectamine 3000 (**Figure 1H**). The low transfection efficiency observed with Lipofectamine 3000 in HeLa is mainly due to the fact that we refrained from using the Enhancer P3000^{TM} reagent during transfection because its use resulted in massive cell death. Although the number of cells expressing GFP is lower after transfection with Lipofectamine 3000 than after transfection with JetPRIME, we did not observe any significant change in the number of cells with altered SC35 localization. Hence, it is very clear that the alteration of SC35 localization observed is due to JetPRIME-mediated transfection and that the distribution of the three altered SC35 localization phenotypes depends on the cell type.

2.2. Induction of aberrant localization of SC35 is independent of the capacity of the transfected DNA to be transcribed.

Since the initial observation was done during transfection of a vector coding for a GFP-tagged protein with JetPRIME we wondered whether the altered localization of SC35 was due to the expression of an exogenous protein. We then transfected various forms of DNA such as salmon sperm DNA (SS DNA), an empty eukaryote vector (pcDNA3) and a bacterial expression vector (pGEX6P2-

10xHIS) in U2OS cells (**Figure 2A-2E**). Irrespective of the transfected DNA, we observed a significant increase in the number of cells with SC35 cytoplasmic granules and enlarged SC35 speckles similar to the ones observed after transfection with the pEGFP-C1 plasmid (**Figure 2F**). Our result indicates that JetPRIME-mediated transfection induces an alteration of SC35 localization independently of the capacity of the DNA transfected to express any protein.

2.3. The SC35 cytoplasmic granules induced by JetPRIME-mediated transfection are distinct from P-bodies and stress granules

There are many membrane-less organelles in the cytoplasm like P-bodies and stress granules that are formed by phase separation of proteins and RNA. Hence, we investigated whether the SC35 cytoplasmic granules we observed could correspond to P-bodies or to stress granules. We therefore stained the cells transfected with GFP plasmids with antibody against DDX6, a marker of P-bodies, and antibody against Tia1, a marker of stress granules, along with antibody against SC35.

Although P-bodies and SC35 cytoplasmic granules looked similar, we did not observe any co-localization between SC35 and DDX6 (**Figure 3A and 3B**) indicating that the SC35 cytoplasmic granules formed due to JetPRIME-mediated transfection are distinct from P-bodies.

Similarly, we did not observe any co-localization between SC35 and Tia1 suggesting SC35 cytoplasmic granules are also distinct from the stress granules. Overall, our results indicate that the SC35 cytoplasmic granules are unique structures in cells that neither correspond to P-bodies nor stress granules (**Figure 3C and 3D**).

2.4. JetPRIME-mediated transfection altered SC35 localization without affecting nuclear speckles

SC35 is widely used as a marker of nuclear speckles, which is a membrane-less organelle formed by the concentration of RNA and proteins involved in RNA metabolism [10]. Because JetPRIME-mediated transfection affects SC35 localization we wondered whether it could also affect the nuclear speckles. Therefore, we tested the localization of SON protein, another nuclear speckle protein that is known to have localization similar to SC35 [11], by co-staining the transfected cells with SON and SC35 antibodies. On the contrary to SC35, SON localization was not affected by JetPRIME-mediated transfection. This suggested that JetPRIME affects the localization of SC35 only without affecting the localization of the nuclear speckles per-se (**Figure 4A and 4B**). To test this hypothesis, we checked the localization of different proteins/antigens such as TMG (2,2,7-trimethylguanosine or m3G), pSer2-RNA pol-II, pSer5-RNA pol-II and phosphorylated SR proteins, known to be involved in RNA processing and to localize to nuclear speckles. It is to be noted that these antigens localize to cytoplasmic granules upon knock-down of factors involved in nucleo-cytoplasmic shuttling [12]. Like for SON protein, we found no difference in the staining pattern of TMG (m3G), pSer2-RNA pol-II and pSer5-RNA pol-II whereas the staining pattern of the phosphorylated SR proteins is modified in the same way as SC35, showing cytoplasmic granules and enlarged speckles in the nucleus when JetPRIME was used for transfection (**Figure 4C, 4D, 4E and 4F**). The phosphorylated SR proteins showed the speckled pattern in the nucleus and also the fine granular pattern in the cytoplasm in un-transfected cells whereas cells transfected using JetPRIME showed enlarged speckled structures in the nucleus and large granules in the cytoplasm as was observed for SC35 (**Figure 4G).** This clearly indicates that JetPRIME-mediated transfection alters the localization of not only SC35 but also phosphorylated SR proteins. However, JetPRIME-mediated transfection does not seem to alter nuclear speckles as a whole since the SC35 and phosphorylated SR protein are still detected in the nuclear speckles.

2.5. The cells with enlarged SC35 speckles caused by JetPRIME-mediated transfection showed compromised transcription

The inhibition of transcription has already been reported to induce enlarged nuclear speckles [9,13–15]. As this phenotype resembles enlarged SC35 speckles (ESS) that was observed upon JetPRIME-mediated transfection (**Figure 1E and Supplementary Figure 1E**) we asked whether this method of transfection had any effect on transcription. The transfected HeLa cells were then stained with antibodies against phospho-Ser2 or phospho-Ser5 form of RNA polymerase II as a readout for transcription since these phosphorylation events occur in the carboxy terminal domain (CTD) of active RNA polymerase II subunit B1 (RPB1) during transcription [16–18]. We found that cells with enlarged SC35 speckles showed indeed, a decrease in the levels of RNA polymerase II Ser2 and Ser5 phosphorylation (**Figure 5A, 5B, 5C and 5D**). This clearly indicates that cells with enlarged SC35 speckles show compromised transcription. Loss of RNA polymerase II Ser2 and Ser5 phosphorylation is known to affect the expression of genes such as XIAP, BRCA1 and FANCD2 [19,20]. As expected, the expression of these genes and as well as the widely used housekeeping genes such as ACTB and GAPDH also drastically decreased upon transfection of GFP plasmid using JetPRIME (**Supplementary Figure 2)**. However, Lipofectamine 3000 mediated transfection also showed a marginal effect on gene expression. It has been previously shown that liposome based transfection formulations have an effect on global gene expression [21]. These results reveal that this transfection method can have an impact on gene expression.

2.6. The JetPRIME-mediated transfection alters splicing pattern of BCL2L1, CASP2 and CASP9 genes

The localization of splicing factors acts as a cue for modulating the alternative splicing. The cytoplasmic localization of phosphorylated SR proteins due to RANBP2 has been shown to alter the splicing pattern [12]. Hence we asked if the JetPRIME-mediated transfection can also alter splicing. We found that the alternative splicing pattern of BCL2L1, CASP2 and CASP9 genes has been altered by transfection of GFP plasmid by using JetPRIME but not by Lipofectamine 3000 (Figure 6). Out of the three genes checked, splicing pattern of CASP2 is dramatically affected while little effect was

observed on splicing of BCL2L1 and CASP9. It is very evident that JetPRIME-mediated transfection can have an impact on alternative splicing.

3. Discussion

Transfection is a powerful method used to decipher the function of a gene. In spite of the extensive use of transfection techniques, very little is known about their side effects. In this study, we reported that the treatment of HeLa or U2OS cells with JetPRIME and plasmid together drastically altered the localization of SC35 protein in HeLa and U2OS cell lines, whereas treatment with only JetPRIME did not have any impact on the localization of SC35. On the contrary, the use of Lipofectamine 3000 in place of JetPRIME did not provoke such alteration. JetPRIME is a cationic

polymer-based reagent whereas Lipofectamine 3000 is a cationic lipid-based transfection reagent. It is possible that the cationic polymer-based methods are inducing such phenotypes.

SC35 is a widely used marker of nuclear speckles and often the dynamics and morphology of nuclear speckles are commented by tracking the SC35 protein~~s~~. In our study, we showed that JetPRIME-mediated transfection of plasmids altered the localization of only SC35 and phosphorylated SR proteins but not that of SON, TMG, pSer2-RNA pol-II and pSer5-RNA pol-II. In other words, the nuclear speckles remained intact whereas only the localization of SC35 and phosphorylated SR proteins are altered. Hence, it is important to follow multiple nuclear speckle markers in order to comment on their morphology and dynamics.

In both Hela and U2OS cells, JetPRIME-mediated transfection triggered the appearance of cytoplasmic granules and enlarged SC35 speckles. The enlarged SC35 speckles is a phenotype similar to the one observed in cells compromised for transcription and RNA splicing [9,22]. And indeed, we observed that JetPRIME also induces a decrease in RNA polymerase II phosphorylation, which implies an inhibition of transcription in these cells. Tight interdependency exists in regulation of chromatin, transcription and RNA splicing. For instance, RNA splicing participates in gene expression through AS-NMD (Alternative splicing resulting in nonsense-mediated mRNA decay) [23]. It is known that approximately 80 % of splicing in HeLa cells is co-transcriptional [22]. The RNA splicing is known to be modulated by transcription machinery by the recruitment of the splicing factors to the transcription site by the Pol II CTD (carboxy-terminal domain) [24]. Reciprocally, chromatin structure and histone signatures have been implicated in regulating gene expression and alternative splicing [25,26]. Consistently, treatment with transcription inhibitor or histone deacetylases inhibitor (trichostatin A) also triggers the accumulation of the splicing factors in splicing speckles and associated with enlargement of splicing speckles [9,27]. Hence it is possible that this side effect on SC35 localization impacts on cellular processes beyond transcription and splicing. Such side effects must therefore be carefully considered when choosing the transfection reagents to study the function of a gene in RNA splicing, gene expression and chromatin organization.

In U2OS cells, JetPRIME-mediated transfection induces the appearance of cytoplasmic granules of SC35, a phenotype similar to that observed upon depletion of factors involved in RAN nucleocytoplasmic transport system such as RAN, Ran-binding protein 2 (RanBP2), RCC1, KPNB1 and TNPO3. The RAN nucleocytoplasmic transport system is necessary for the sequential entry of a set of RNA processing components into the nucleus. [12]. While RAN nucleocytoplasmic transport system factors such as RAN and RanBP2 are necessary for nuclear entry of hnRNP-A1, phospho-Ser2 RNA polymerase II, phospho-Ser5 RNA polymerase II, SC35 and phosphorylated-SR proteins; TNPO3 is important specifically for nuclear entry of SR proteins such as SC35 [12,28,29]. Since we observed cytoplasmic granules of only SC35 and p-SR, it is possible that JetPRIME-mediated transfection affects the TNPO3 protein or a related pathway.

Similarly to our work, different transfection reagents have been shown to induce different side effects in different cells. For instance, the two lipid-based reagents, Lipofectamine and FuGENE® HD mediated plasmid transfection activates interferon signalling pathway at mRNA and protein level in HeLa cells but not in HEK293T cells [7]. Similarly, Lipofectamine is reported to trigger type I interferon response in macrophages whereas such side effect is unseen with another transfection reagent, Xfect (polymer-based transfection reagent). Also, the side effect is seen only with macrophage-like cells but not in 293T or L929 cells [30]. These reports give a novel example where the lipid-based transfection systems are unsuitable in studies related to innate immunity where the lipid signalling plays an important role. These works also suggest the use of the right combination of cell line and transfection reagents to study the function of a gene.

Various transfection reagents have been reported to be the source of various side effects. In most cases, these artifacts can often be misjudged as a phenotype induced by the overexpression or knockdown of the gene of interest. These mis-interpretations can be avoided by using proper negative control to the transfection experiments. Many side effects caused by transfection reagents are cell-type and transfection reagent specific. Hence these types of studies reporting artifacts strongly recommend the use of right transfection reagent for the right cell lines for answering the question of interest. Such studies also put forth the scope of improvement and innovation for the companies that

manufacture transfection reagents, by taking into account of such artifacts while developing the new transfection reagents.

4. Materials and methods

4.1. Cell culture and transient transfection

U2OS cells and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM)-GlutaMAX ™ -I (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS)(GE Healthcare) and 1% penicillin-streptomycin (GE Healthcare). For transfection of pEGFP-C1 plasmids, cells were grown till 70 % confluence and were transfected using JetPRIME® (Polyplus-transfection S.A, Illkirch, France) or Lipofectamine-3000 (ThermoFisher scientific) transfection reagents according to the manufacturer's instructions. Briefly, for JetPRIME-mediated transfection in a 12 well format, 0.5 µg of plasmid was diluted into 100µl of jetPRIME® buffer and mixed well before adding 1µl of jetPRIME® reagent. This mixture was incubated in room temperature for 10 minutes and added to the cells in serum containing DMEM medium. The medium was changed after 4 hours of transfection and grown further for 20 hours before processing the cells. In case of Lipofectamine 3000 mediated transfection, the lipofectamine mix and plasmid mix were prepared separately and then finally mixed together and incubated for 5 minutes at room temperature before adding to the cells in Opti-MEM® Medium. The lipofectamine mix was prepared by diluting 1.5µl of Lipofectamine® 3000 reagent in 50µl of Opti-MEM® Medium. The plasmid mix was prepared by diluting 0.5 µg of plasmid and 1.0 µl of P3000™ Reagent in 50µl of Opti-MEM® Medium. The P3000™ Reagent was avoided when transfecting in HeLa cells. The medium was changed after 4 hours of transfection and grown further for 20 hours before processing the cells.

4.2. Immunofluorescence staining

The cells grown on coverslips were fixed after 24 hours of transfection with 100% methanol (-20ºC for 4 minutes). The cells were then blocked with 5% FBS in PBS and incubated with the primary antibody in blocking solution for 1 hour at room temperature. Cells were washed thrice in

PBS and incubated with secondary antibody in blocking buffer for one hour at room temperature. The cells were then washed and the DNA was stained using DAPI (Thermo Fisher Scientific). Finally, the coverslips were mounted using ProLong® Gold antifade reagent (Thermo Fisher Scientific).

The following primary antibodies were used: Mouse anti-SC35 (S4045, Sigma; 1:2000), Goat anti-TIA1 (sc-1751, Santa Cruz; 1:200), Rabbit anti-DDX6 (NB200-192, Novus biologicals; 1:2000), Mouse anti-phospho-SR proteins (16H3E8, Thermo Fisher Scientific; 1:50), Rat anti-phospho-Ser2-RNA pol II (3E10; 1:100), Rat anti-phospho-Ser5-RNA pol II (3E8; 1:100), Mouse anti-snRNA, TMG (m3G) (Oncogene; 1:100), Rabbit anti-SON (HPA023535, Sigma-Aldrich. 1:1000), Chicken anti-GFP (GFP-1020, Aves Labs; 1/500)

The following secondary antibodies were used: Alexa 546 conjugated Goat anti-mouse, Alexa 647 conjugated Goat anti-mouse, Alexa 546 conjugated Goat anti-Rabbit, Alexa 555 conjugated Goat anti-Rat, Alexa 546 conjugated Donkey anti-Goat, FITC conjugated Donkey anti-Chicken, Alexa 555 conjugated Goat anti-Chicken (Thermo Fisher Scientific), Cy5 conjugated Donkey anti-Mouse (Jackson Immuno Research).

4.3. Deltavision microscopy

The images were taken using a deltavision (Olympus IX7) microscope equipped with 60X oil immersion objective, CoolSnapHQ Princeton BW camera and image acquisition software MetaVue (Molecular Devices, Inc.). All the images were processed using the FIJI (ImageJ) software.

4.4. RNA isolation, RT-PCR and RT-qPCR

HeLa cells were transfected with or without pEGFP-C1 plasmid using JetPRIME or Lipofectamine 3000 and grown for 24 hours. The total RNA was isolated with the RNeasy Plus Mini Kit (QIAGEN- Cat No./ID: 74134) as per the manufacturer's protocol. While isolating total RNA, on-column digestion of DNA was additionally performed using RNase-Free DNase Set (QIAGEN - Cat No./ID: 79254). The gene expression was analyzed by RT-qPCR and splicing pattern was analyzed by RT-PCR. For both RT-PCR and RT-qPCR experiments, cDNA was synthesized using (Invitrogen ™) SuperScript ™ III Reverse Transcriptase. Briefly, 500 ng total RNA was reverse transcribed in a 20µl reaction containing 200 units of Superscript III reverse transcriptase, 40 units of RNAseOUT™ recombinant ribonuclease inhibitor, 1X First strand buffer, 5mM of DTT, 0.5mM of dNTPs, 1.25µM of oligo-dT (Sigma-Aldrich) and 125ng of random primers (Life Technologies). The synthesized cDNA was diluted 6 times and used for RT-PCR.

RT-PCR reactions were carried out using 2µl of diluted cDNA in a 30µl reaction containing 1X GoTaq® Flexi Buffer, 1.5mM of $MgCl_2$, 0.2mM of dNTPs, 0.5µM primers and 0.5 units of GoTaq® DNA Polymerase. The PCR reactions were carried out in the following condition: 95°C for 1 minute followed by 30 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds and finally at 72°C for 1 minute. The primers used for RT-PCR are as follows: BCL2L1 (F: CAGTAAAGCAAGCGCTGAGG and R: AAGAGTGAGCCCAGCAGAAC); CASP2 (F: GCTCTTTGACAACGCCAACT and R: GGCATAGCCGCATATCATGT); CASP9 (F : CGGGCAGGCTCTGGATCTC and R-1 : CCAGCACCATTTTCTTGG; R-2 : TGGTCTTTCTGCTCGACA)

RT-qPCR was carried out using *Power* SYBR™ Green PCR Master Mix (Applied Biosystems) on a QuantStudio 7 Flex Real-Time PCR Systems. Threshold cycle (C_T) values for triplicate qPCRs were determined using QuantStudio Software v1.3, and relative quantification of the transcripts were determined through normalization to 18s rRNA. The primers used for RT-qPCR are as follows: XIAP (F: TGGGACATGGATATACTCAGTTAACAA and R: GTTAGCCCTCCTCCACAGTGAA); BRCA1 (F: GCCAAGGCAAGATCTAGAGG and R: GTTGCCAACACGAGCTGA); FANCD2 (F: CCCAGAACTGATCAACTCTCCT and R:

CCATCATCACACGGAAGAAA); 18S rRNA (FP: GTAACCCGTTGAACCCCATT and R: CCATCCAATCGGTAGTAGCG); Beta-actin (ACTB) (F: CCAACCGCGAGAAGATGA and R: CCAGAGGCGTACAGGGATAG); GAPDH (F: GAAGGTGAAGGTCGGAGTC and R/ GAAGATGGTGATGGGATTTC)

4.5. Statistical analysis

All the experiments were performed in independent biological triplicates and all statistical analyses were performed using two-tailed pairwise student's t-tests. At least 100 cells were quantified for each experimental condition.

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Figure legends

Figure 1: Transfection of GFP plasmid using JetPRIME affects the localization of SC35 in U2OS and HeLa cells.

U2OS cells were transfected using JetPRIME or Lipofectamine 3000 with (B and D) or without (A and C) GFP-plasmid and then grown for 24 hours at 37°C. The cells were stained with anti-SC35 and anti-GFP antibodies and DNA was visualized with DAPI. The last panel in A, B, C and D shows the merged image excluding the GFP channel. (E) Magnification of cells corresponding to the cells numbered in A and B showing examples of cells with normal and affected SC35 localization as indicated. (F) Graph showing the quantification of U2OS cells expressing GFP in A, B, C and D. (G) Graph showing the quantification of U2OS cells with altered SC35 localization in A, B, C and D. (H) Graph showing the quantification of HeLa cells expressing GFP in A, B, C and D of Supplementary Figure 1. (I) Graph showing the quantification of HeLa cells with altered SC35 localization in A, B, C and D of Supplementary Figure 1. At least 100 cells were counted for each experimental condition. [Scale bar = 10 μm]

Figure 2: JetPRIME affects SC35 localization independently of the exogenous gene expression by the transfected vector

(A-E) U2OS cells were transfected with 1μg of DNA/plasmids using JetPRIME and then grown for 24 hours. The cells were stained with anti-SC35 and anti-GFP antibodies and DNA was visualized with DAPI. The last panel in A, B, C, D and E shows the merged image excluding the GFP channel. (F) Left Panel: Graph showing the quantification of U2OS cells with altered SC35 localization in A,

B, C, D and E. Right Panel: The table indicates the P values of the left panel in F. At least 100 cells were counted for each experimental condition. [Scale bar = 10 μm].

Figure 3: The JetPRIME induced SC35 cytoplasmic granules are distinct from P-bodies and stress granules

(A) U2OS cells were transfected with pEGFP-C1 plasmid using JetPRIME and stained with anti-GFP, anti-SC35 and anti-DD6 antibodies and DNA was visualized with DAPI. (B) Magnification of boxed and numbered region of cells in A which show the localization of P-bodies and SC35. The arrows indicate the cytoplasmic granules of SC35. (C) U2OS cells were transfected with pEGFP-C1 plasmid using JetPRIME and stained with anti-GFP, anti-SC35 and anti-Tia1 antibodies and DNA was visualized with DAPI. (D) Magnification of boxed region of the cell in C which show the localization of stress granules and SC35. The arrow indicates the stress granules and the arrowhead indicates the cytoplasmic granules of SC35. [Scale bar = 10 μm].

Figure 4: JetPRIME-mediated transfection altered only the SC35 localization but not the nuclear speckles

(A) U2OS cells were transfected with pEGFP-C1 plasmid using JetPRIME and stained with anti-GFP, anti-SC35 and anti-SON antibodies and DNA was visualized with DAPI. (B) Magnification of boxed region of the cell in A which show the localization of SON and SC35 proteins. The arrows indicate the cytoplasmic granules of SC35. (C-F) U2OS cells were transfected with pEGFP-C1 plasmid using JetPRIME and stained with anti-GFP, and anti-m3G (C) or anti-pS2-RNA pol-II (D) or anti-pS5-RNA pol-II (E) or anti-p-SR (F) antibodies. DNA was visualized with DAPI. (G) Magnification of cells numbered in F. Arrowhead and arrow indicate the fine and large cytoplasmic granules of phosphorylated SR proteins. [Scale bar = 10 μm].

Figure 5: The cells with enlarged SC35 speckles by JetPRIME showed compromised transcription

(A) U2OS cells were transfected with pEGFP-C1 plasmid using JetPRIME and stained with anti-GFP, anti-SC35 and anti-pS2-RNA pol-II antibodies and DNA was visualized with DAPI. (B) Magnification of cells numbered in A which show the decrease in levels of pS2-RNA pol-II staining in cells with enlarged SC35 speckles. (C) U2OS cells were transfected with pEGFP-C1 plasmid using JetPRIME and stained with anti-GFP, anti-SC35 and anti-pS5-RNA pol-II antibodies and DNA was visualized with DAPI. (D) Magnification of cells numbered in C which show the decrease in levels of pS5-RNA pol-II staining in cells with enlarged SC35 speckles. [Scale bar = 10 μm].

Figure 6: The JetPRIME-mediated transfection modifies alternative splicing pattern

HeLa cells were transfected using JetPRIME or Lipofectamine 3000 with or without GFP-plasmid and then grown for 24 hours at 37°C. RT-PCR were performed to follow alternative splicing of three pre-mRNAs, BCL2L1 (A, D), CASP2 (B, E) and CASP9 (C, F). (A-C) show 6% acrylamide gels of RT-PCR products and (D-F) show the quantification of 'percent spliced index' from the gel images.

Declaration of competing interests
The authors declare no conflict of interest.

Highlights

- JetPRIME-mediated transfection alters the localization of the splicing protein, SC35, causing enlarged SC35 speckles and SC35 cytoplasmic granules.
- SC35 cytoplasmic granules are distinct from P-bodies and stress granules.
- This method affects SC35 and p-SR proteins without affecting nuclear speckles.
- JetPRIME-mediated transfection can also affect gene expression and alternative splicing.

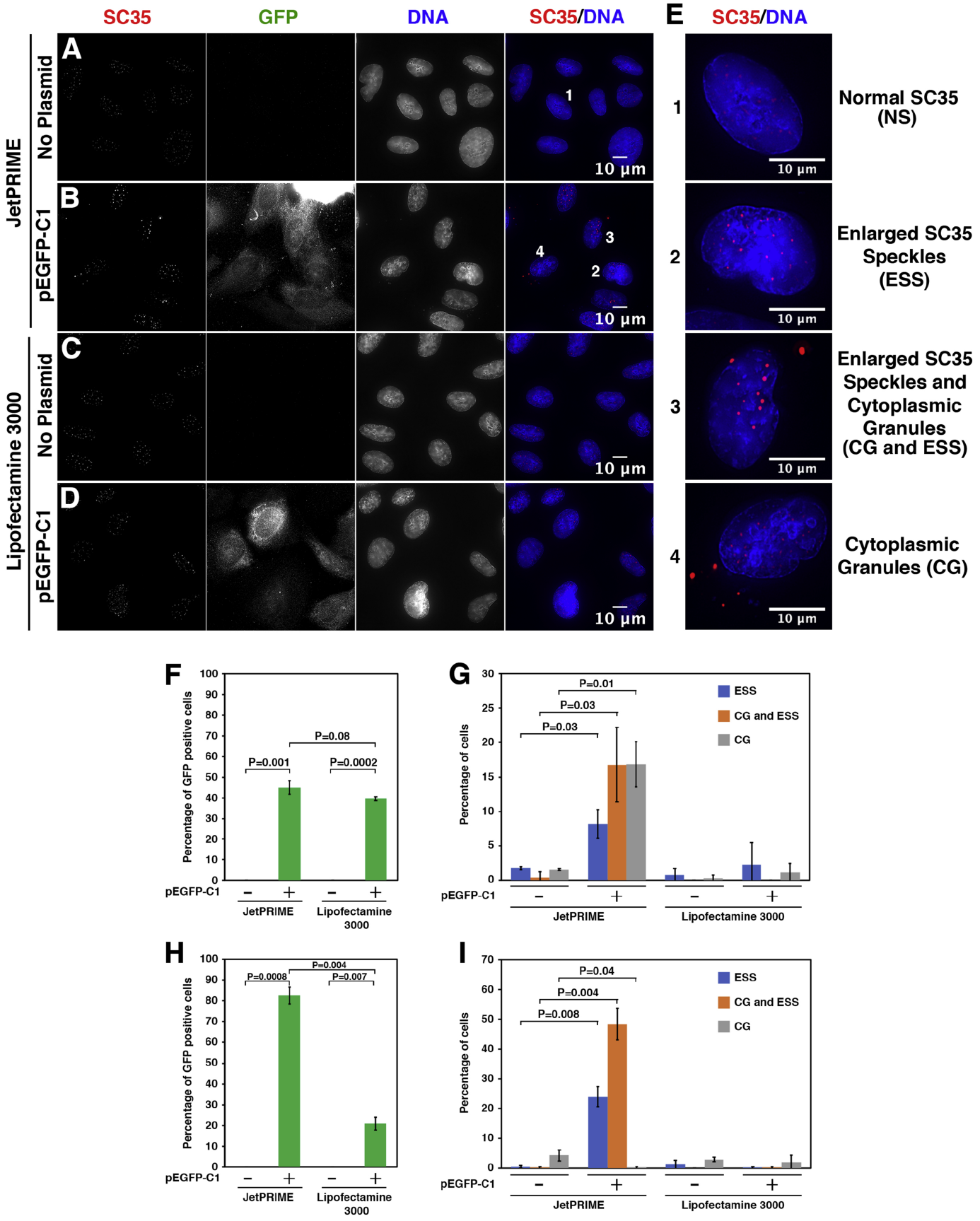
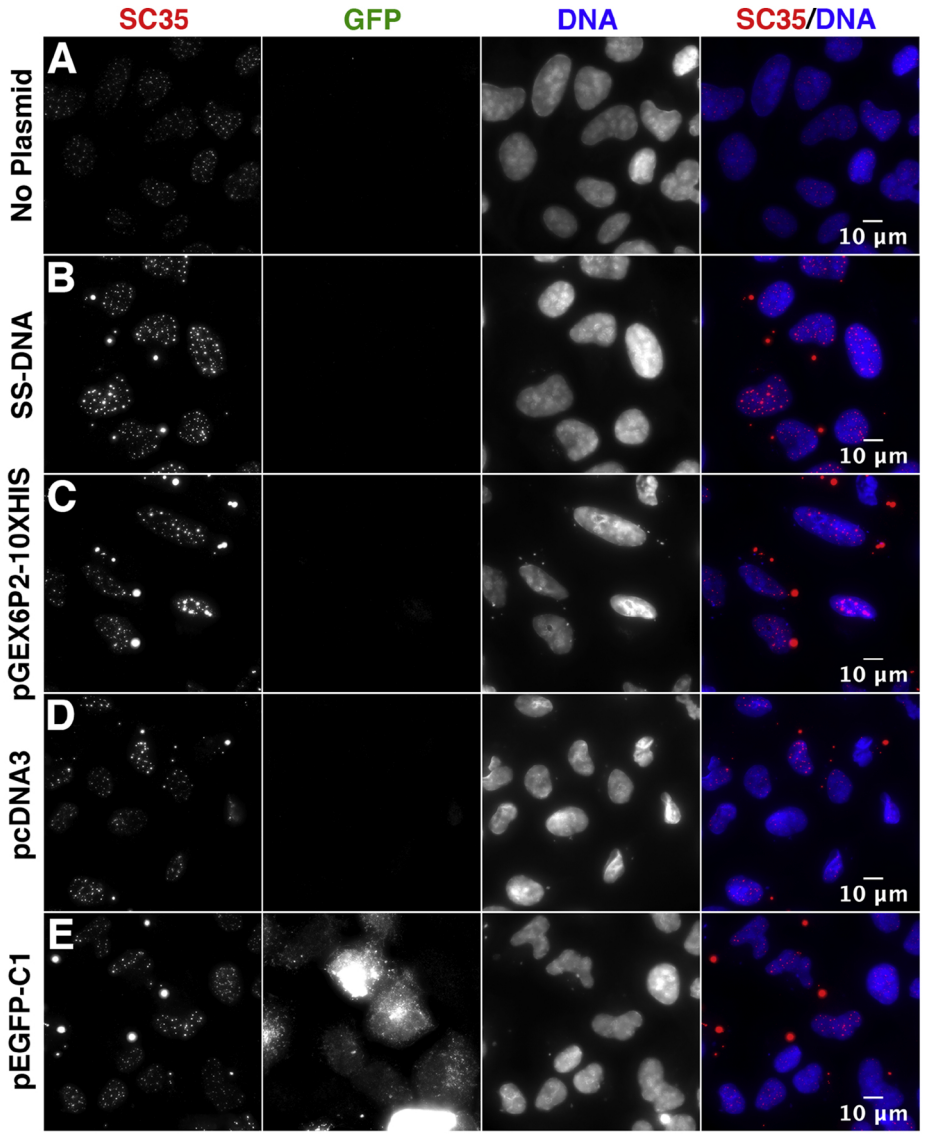
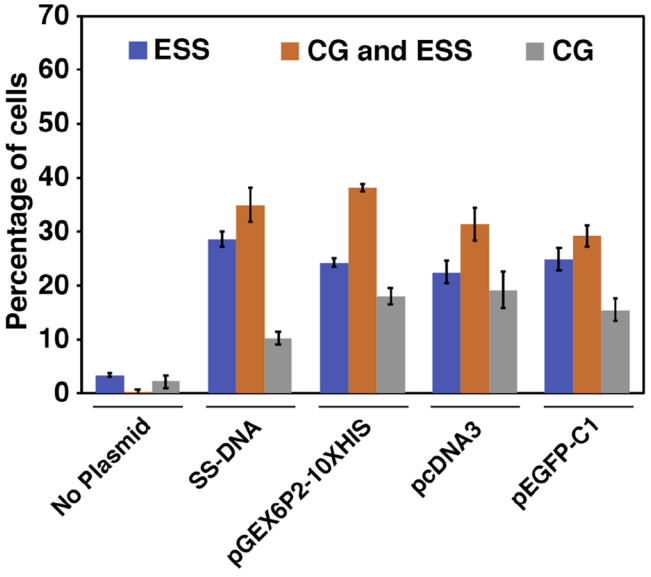

SC35
GFP
DNA
SC35/DNA
E
SC35/DNA
JetPRIME
Lipofectamine 3000
No Plasmid
pEGFP-C1
A
B
C
D
1
2
3
4
10 µm
Normal SC35 (NS)
Enlarged SC35 Speckles (ESS)
Enlarged SC35 Speckles and Cytoplasmic Granules (CG and ESS)
Cytoplasmic Granules (CG)
F
Percentage of GFP positive cells
P=0.08
P=0.001
P=0.0002
pEGFP-C1
JetPRIME
Lipofectamine 3000
G
Percentage of cells
P=0.01
P=0.03
P=0.03
ESS
CG and ESS
CG
H
P=0.004
P=0.0008
P=0.007
I
P=0.04
P=0.004
P=0.008

Figure 1



F



P values

ESS	CG and ESS	CG	
0.0011	0.0027	0.0213	SS DNA
0.0006	0.0001	0.0031	pGEX6P2-10XHIS
0.0050	0.0025	0.0235	pcDNA3
0.0026	0.0010	0.0191	pEGFP-C1
No Plasmid			

Figure 2

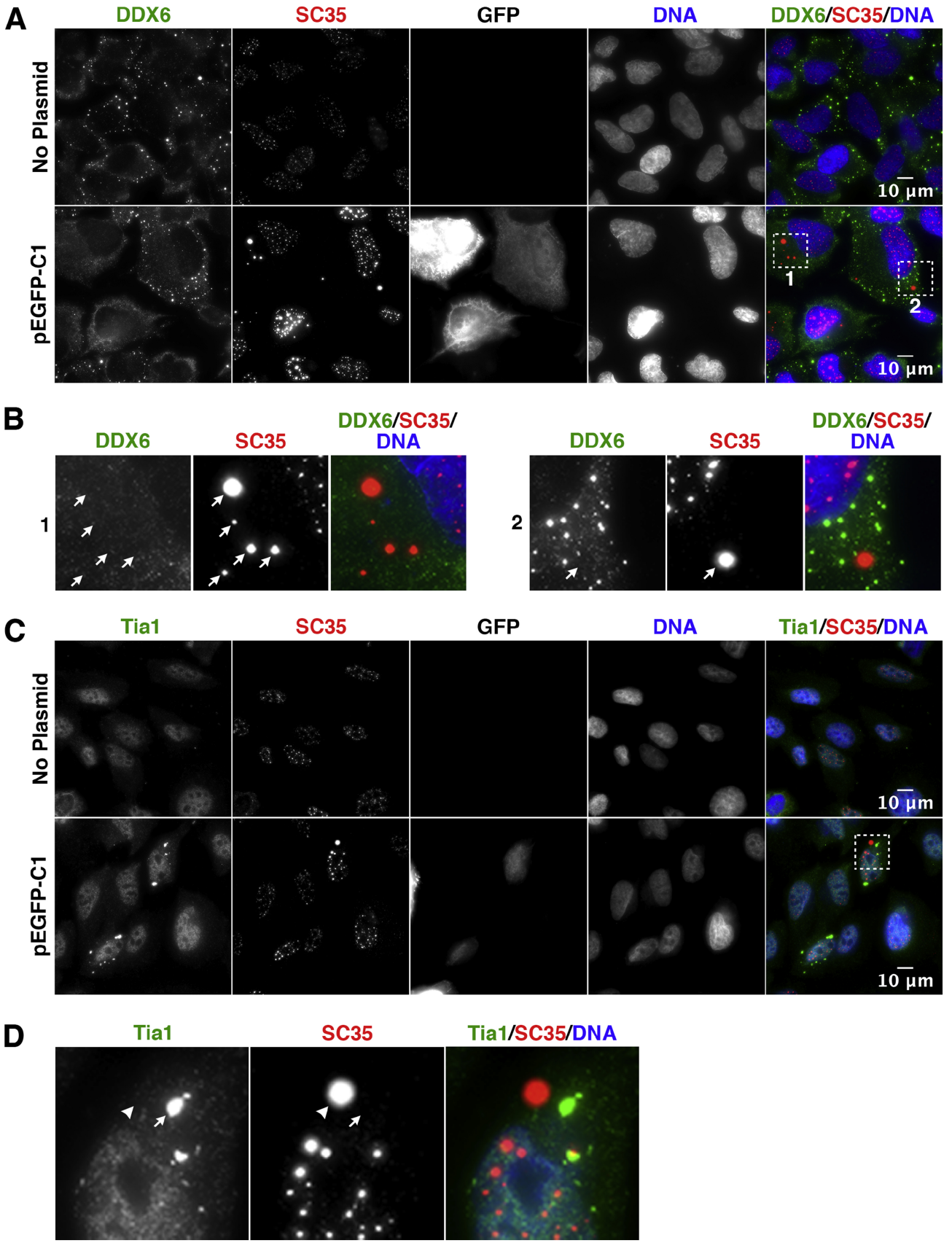

A
DDX6
SC35
GFP
DNA
DDX6/SC35/DNA
No Plasmid
pEGFP-C1
10 μm
1
2
B
DDX6
SC35
DDX6/SC35/
DNA
C
Tia1
Tia1/SC35/DNA
D

Figure 3

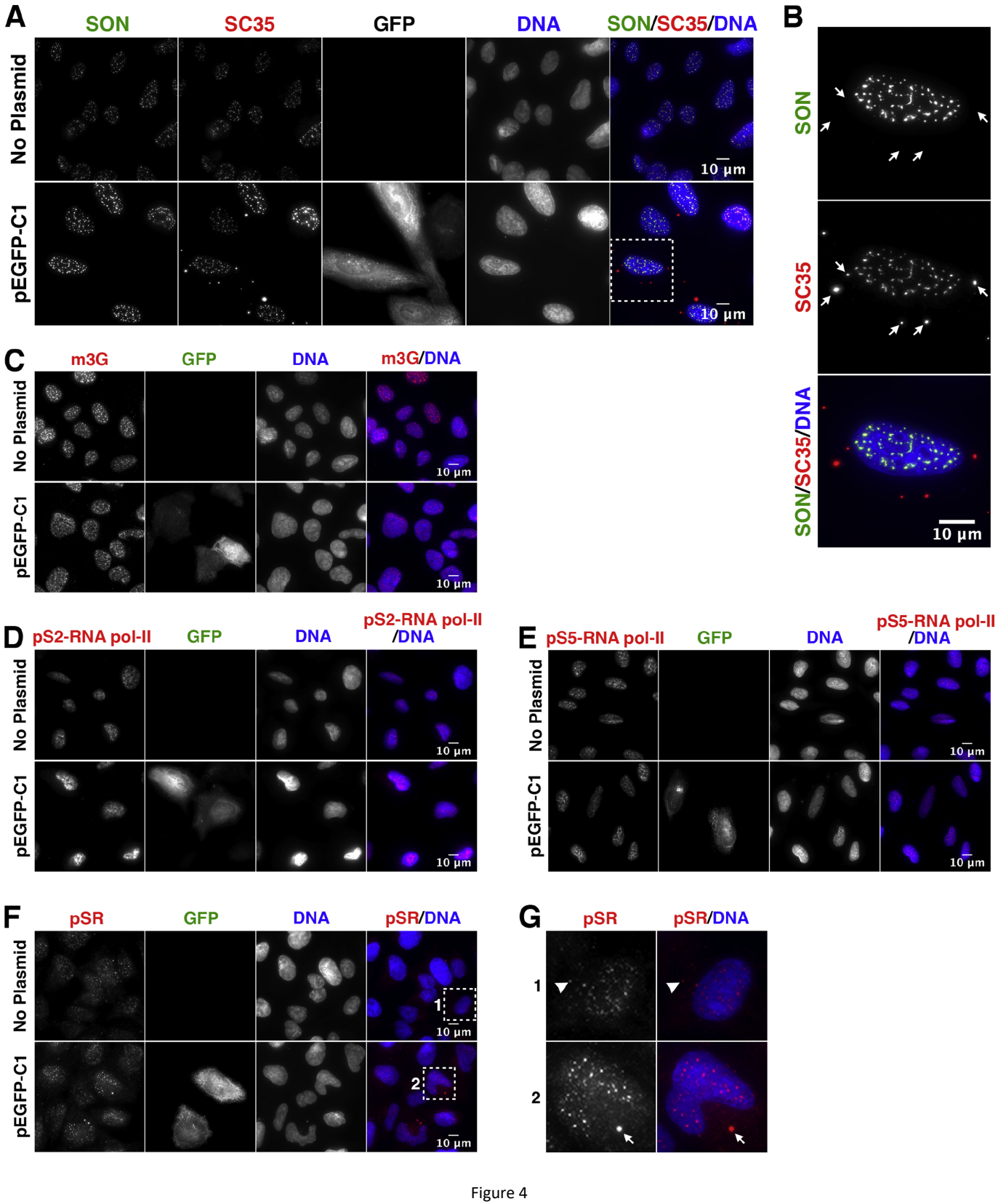

A
SON
SC35
GFP
DNA
SON/SC35/DNA
No Plasmid
pEGFP-C1
10 μm
B
SON
SC35
SON/SC35/DNA
10 μm
C
m3G
GFP
DNA
m3G/DNA
No Plasmid
pEGFP-C1
10 μm
D
pS2-RNA pol-II
GFP
DNA
pS2-RNA pol-II /DNA
No Plasmid
pEGFP-C1
10 μm
E
pS5-RNA pol-II
GFP
DNA
pS5-RNA pol-II /DNA
No Plasmid
pEGFP-C1
10 μm
F
pSR
GFP
DNA
pSR/DNA
No Plasmid
pEGFP-C1
1
2
10 μm
G
pSR
pSR/DNA
1
2

Figure 4

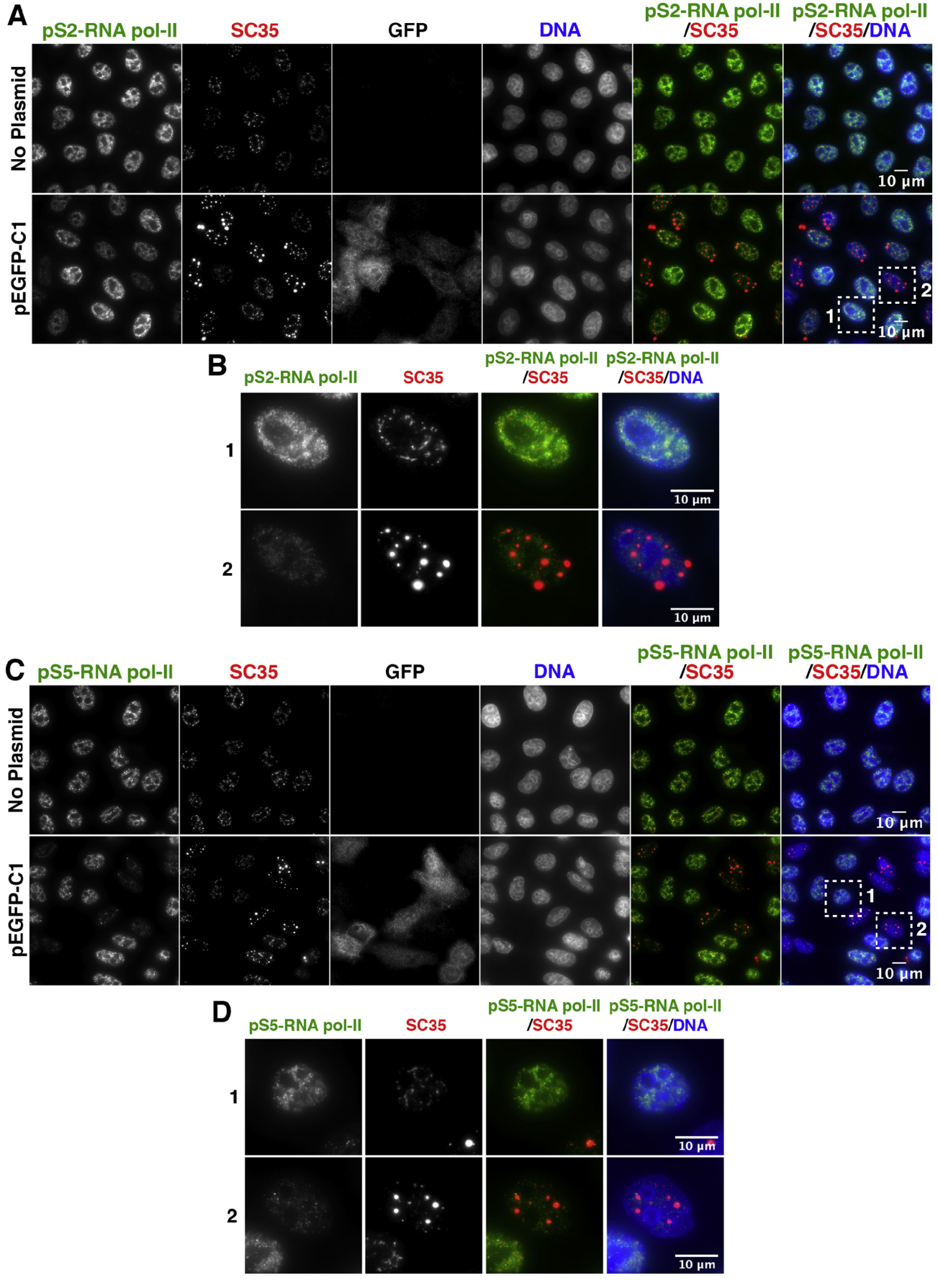

A
pS2-RNA pol-II
SC35
GFP
DNA
pS2-RNA pol-II /SC35
pS2-RNA pol-II /SC35/DNA
No Plasmid
pEGFP-C1
10 µm
1
2
B
pS2-RNA pol-II
SC35
pS2-RNA pol-II /SC35
pS2-RNA pol-II /SC35/DNA
C
pS5-RNA pol-II
SC35
GFP
DNA
pS5-RNA pol-II /SC35
pS5-RNA pol-II /SC35/DNA
D
pS5-RNA pol-II
SC35
pS5-RNA pol-II /SC35
pS5-RNA pol-II /SC35/DNA

Figure 5

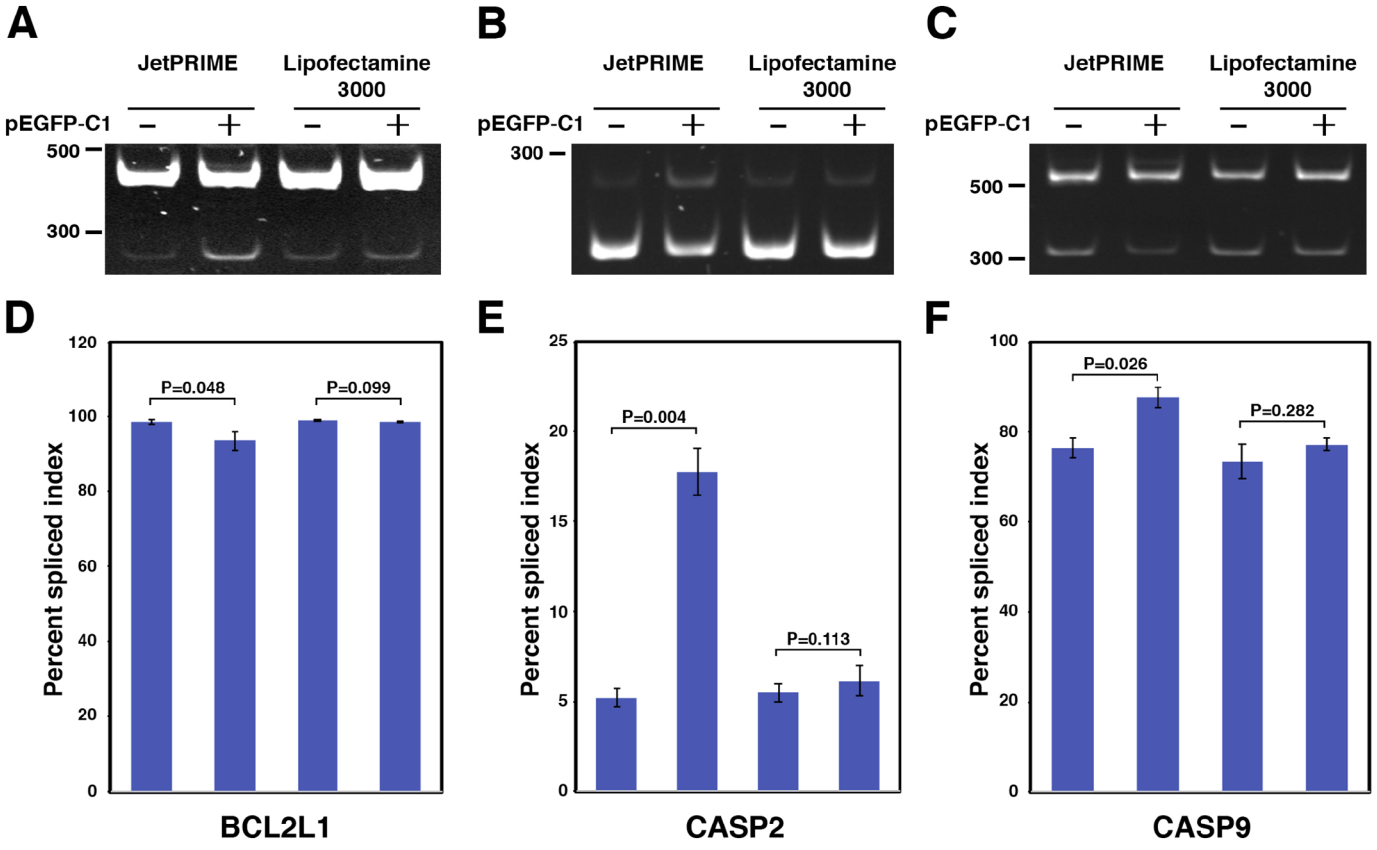
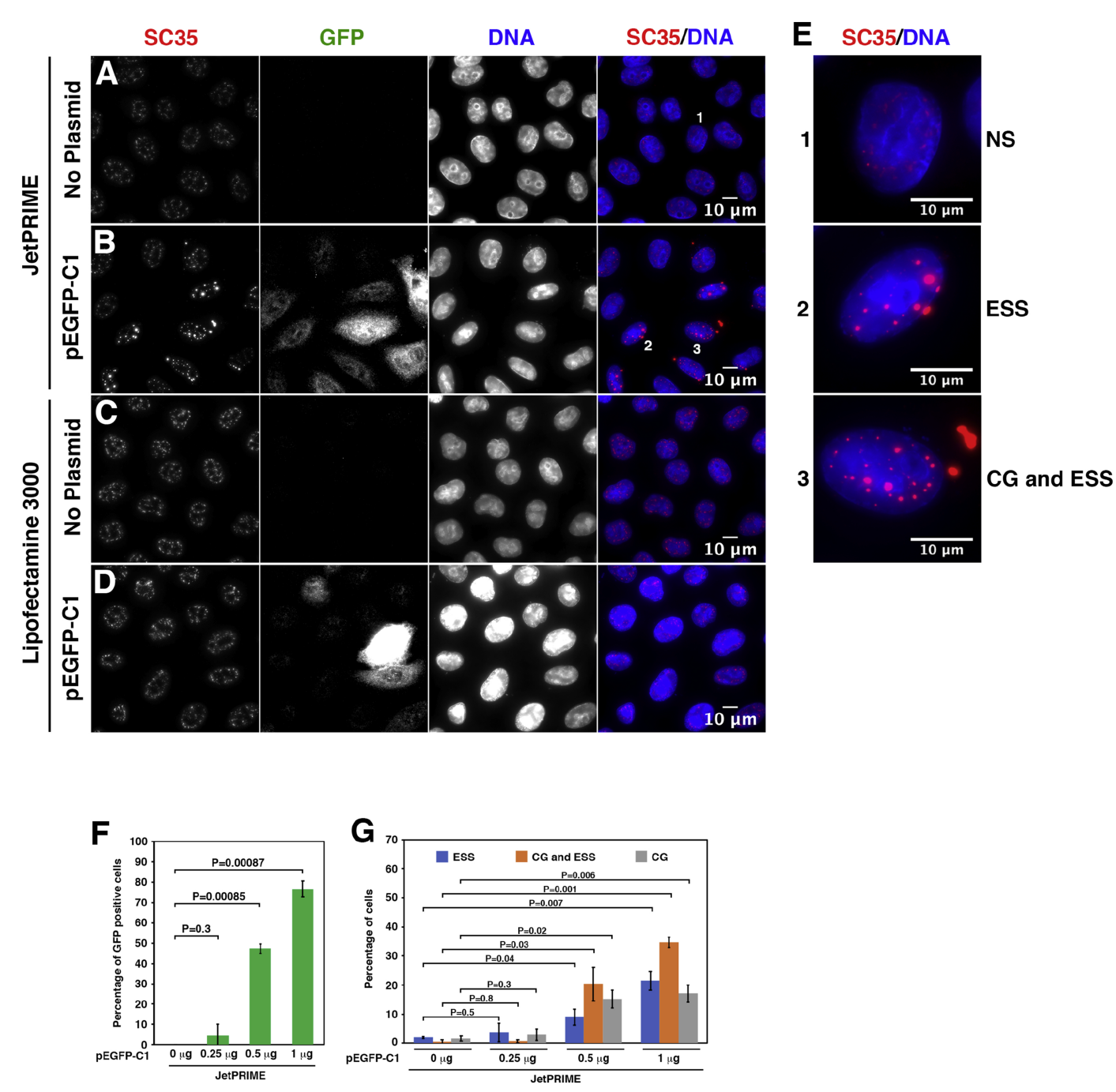
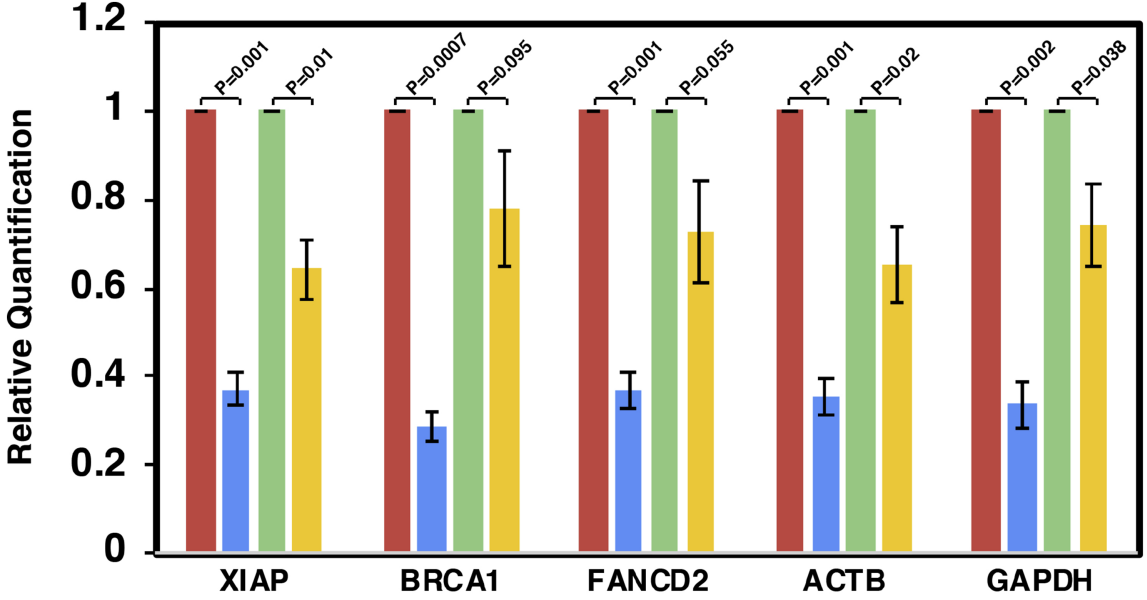

A
JetPRIME
Lipofectamine 3000
pEGFP-C1
–
+
–
+
500
300
B
JetPRIME
Lipofectamine 3000
pEGFP-C1
–
+
–
+
300
C
JetPRIME
Lipofectamine 3000
pEGFP-C1
–
+
–
+
500
300
D
Percent spliced index
120
100
80
60
40
20
0
P=0.048
P=0.099
BCL2L1
E
Percent spliced index
25
20
15
10
5
0
P=0.004
P=0.113
CASP2
F
Percent spliced index
100
80
60
40
20
0
P=0.026
P=0.282
CASP9

Figure 6


SC35
GFP
DNA
SC35/DNA
JetPRIME
Lipofectamine 3000
No Plasmid
pEGFP-C1
A
B
C
D
1
2
3
10 µm
E
SC35/DNA
1
NS
2
ESS
3
CG and ESS
10 µm

F
Percentage of GFP positive cells
P=0.00087
P=0.00085
P=0.3
pEGFP-C1
0 µg
0.25 µg
0.5 µg
1 µg
JetPRIME
G
ESS
CG and ESS
CG
Percentage of cells
P=0.006
P=0.001
P=0.007
P=0.02
P=0.03
P=0.04
P=0.3
P=0.8
P=0.5
pEGFP-C1
0 µg
0.25 µg
0.5 µg
1 µg
JetPRIME

Figure 7



	pEGFP-C1	
JetPRIME	-	
	+	
Lipofectamine 3000	-	
	+	

Figure 8