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Changes in Fluorescence Recovery After Photobleaching (FRAP) as an indicator of SOX9 transcription factor activity☆

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ABSTRACT

Cells respond to their environment via an intricate cellular signaling network, directing cell fate. Changes in cell fate are characterized by changes in gene transcription, dictated by (master) transcription factor activity. SOX9 is the master transcription factor for chondrocyte development. Its impaired function is implicated in osteoarthritis and growth disorders, such as dwarfism. However, the factors regulating SOX9 transcriptional activity are not yet fully mapped. Current methods to study transcription factor activity are indirect and largely limited to quantification of SOX9 target gene and protein expression levels after several hours or days of stimulation, leading to poor temporal resolution. We used Fluorescence Recovery After Photobleaching (FRAP) to study the mobility of SOX9 and correlated the changes in mobility to changes in its transcriptional activity by cross-validating with chromatin immunoprecipitation and qPCR. We show that using FRAP, we can quantify the changes in SOX9 mobility on short time scales as an indication of transcriptional activity, which correlated to changes of SOX9 DNA-binding and long-term target gene expression.

1. Introduction

SOX9 is the master transcription factor of cartilage formation during the differentiation of mesenchymal stem cells into chondrocytes. SOX9 controls several processes during development, differentiation, disease and tissue homeostasis, and is under control of several signaling networks, including hedgehog, notch, WNT/β-catenin and TGF-β signaling. Its differential regulation is implicated in many diseases, including dwarfism and other growth disorders, and in cartilage pathophysiology, such as osteoarthritis (OA) [1].

The regulation of SOX9 transcriptional activity is not yet fully mapped. In contrast, SOX9 expression patterns have been described in many tissue types, such as pancreas, intestine, lung, liver, cartilage and testis etc. [1,2]. TGF-β, BMP7 and FGF positively regulate SOX9 activity, whereas WNT3a, IL1β and notch signaling negatively regulate its activity [3]. During OA, SOX9 activity and expression is reduced [4]. We have previously shown that loss of ACAN and COL2A1 expression precedes loss of SOX9 expression, indicating that during OA SOX9 activity is not solely reduced by a lack of SOX9 expression [5]. This indicates that there are factors regulating SOX9 protein activity. In this paper, we tested whether factors that are known to be differentially regulated in OA can directly influence SOX9 transcriptional activity. For this we chose a positive regulator of SOX9, BMP7, and two negative regulators of cartilage homeostasis, IL1β and WNT3a.

Cartilage development and homeostasis is traditionally measured in terms of expression of SOX9 and its target genes, collagen 2 and aggrecan, at the mRNA level. In chondrocytes, BMP7 stimulates SOX9 target genes and thereby the production of chondrocyte specific extracellular matrix genes such as proteoglycan and collagens [6–9] and is clinically approved for cartilage and bone tissue regeneration [10,11]. WNT3a is a prototypical, well-characterized activator of the canonical WNT/β-catenin pathway [12], downregulates cartilage matrix genes and has a well-documented role in osteoarthritis pathology [13,14]. Among several pro-inflammatory cytokines, IL1β plays a keyrole in cartilage pathology [15]. IL1β is a potent inhibitor of the chondrocyte phenotype by downregulating *SOX9*, *COL2A1* and *ACAN* mRNA expression [16,17]. Although much is known about downstream regulation of SOX9 target gene expression, the upstream factors regulating SOX9 protein activity are not yet fully mapped.

Currently, the activity of SOX9 is studied indirectly and on the long-term, using qPCR, promotor reporter assays, western blot or immunofluorescence by measuring the target gene or protein expression levels

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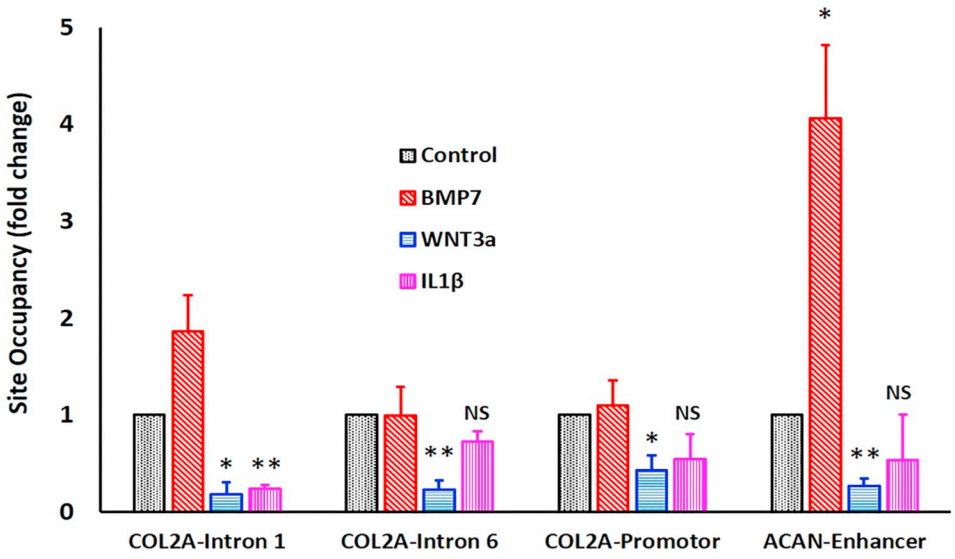

Fig. 1. ChIP-qPCR shows that the external stimulation of C-20/A4 cells for 1 h directly influenced SOX9 binding to DNA. BMP7 (100 ng/ml, red bars) treatment increases SOX9 occupancy at ACAN-Enhancer sites and tended to increase at COL2A-Intron 1, while COL2A-Intron-6 and COL2A-Promotor sites remain unchanged. WNT3a (10 ng/ml, blue bars) and IL1β (10 ng/ml, pink bars) treatments reduce SOX9 occupancy at all tested SOX9 binding sites. Data expressed as mean fold-change of two independent experiments. Unpaired, 2-tailed, Student *t*-test was performed between control and treatments using respective 2nd $\Delta\Delta C_t$ (fold change) values. $^*p < 0.05$, $^{**}p < 0.01$.

and correlating this to the transcription factor activity [18]. However, these traditional methods have some limitations. For example, mRNA expression does not always result in subsequent protein expression and protein expression does not always correspond to protein activity [5]. Secondly, although these methods provide effective qualitative and quantitative information on gene and protein expression, information on transcription factor dynamics and activity is limited. Thirdly, often hundreds to thousands of cells are used for these experiments, limiting the use of these methods for investigating transcription factor activity in rare tissue samples. These limitations necessitate the need of a new method that enables the study of the activity of transcription factors directly.

We used FRAP to study the real-time dynamics of SOX9 with spatiotemporal resolution in response to a variety of extracellular signals. We applied the principle that the mobility of nuclear receptors can be correlated to their activity level. In theory, an active transcription factor is transiently bound to DNA, rendering it relatively immobile, whereas an unbound transcription factor will be more highly mobile, and this can be quantified by FRAP. Although FRAP has already been used to study the mobility of transcription factors in steady-state situations, mobility was not previously correlated to changes in DNA binding and target gene expression levels in response to changes in the extracellular environment. Fluorescence Correlation Spectroscopy (FCS) and Single Molecule Microscopy (SMM) are other direct methods, which can probe transcription factor mobility. However, they require expensive and complex instrumentation and analysis methods [19].

Most methods currently applied to study the activity of transcription factors fail to capture the immediate changes of SOX9 activity following stimulation. To bridge this gap, we applied FRAP as a fast, relatively simple, less expensive and more direct method, to study the immediate changes of transcriptional activity in response to external stimuli. FRAP provides quantitative measures of protein dynamics, such as immobile fraction (IF, fraction bound to DNA), half-time to recover ($t_{½}$), and the ratio of the fast diffusing population (unbound transcription factor) to slow diffusing population (interaction at the binding site) (A_1/A_2) of fluorescent moieties [19].

We measured SOX9 transcriptional activity by FRAP in the chondrocyte cell line C-20/A4 [20] both in the presence and absence of known regulators of SOX9, such as BMP7, WNT3a and IL1β. In this paper, we show that the stimulation of C-20/A4 cells by these factors immediately and directly changes SOX9 binding with DNA and transcriptional activity and that these changes can be detected in living single cells by FRAP.

2. Results

2.1. External stimulation of C-20/A4 cells changes SOX9 binding to target gene promotors

To test our hypothesis that the mobility of transcription factors is linked to their activity and that this can be measured by FRAP, we defined two research questions: 1) Can SOX9 directly respond to treatment of cells with external stimuli? and 2) Can we apply FRAP as a direct readout of SOX9 transcriptional activity in response to external stimuli on short time scales?

Our first research question is whether the activity of SOX9, is directly influenced by external stimuli. To answer this, Chromatin Immunoprecipitation (ChIP) was applied. ChIP is used to detect or quantify protein-DNA interactions at specific sites on the DNA. In chondrocytes, SOX9 has several specific binding sites on DNA [21–23]. We treated SOX9-mGFP transfected C-20/A4 cells for 1 h with various factors that are known to either function as an anabolic factor in articular cartilage, BMP7, or as a catabolic factor, WNT3a, or as an inflammatory factor, IL1β.

BMP7 enriched SOX9 occupancy at the *COL2A* intron-1 and *ACAN promotor sites*, while site occupancy at *COL2A* intron-6 and *COL2A* promoter sites remained unchanged (Fig. 1, red bars). Treatment with WNT3a reduced SOX9 binding in all target sites tested, (Fig. 1, blue bars). This is expected, as in the presence of WNT3a, mRNA expression levels of SOX9 target genes, such as *COL2A* and *ACAN*, are downregulated [13,24]. Treatment by IL1β resulted in reduced SOX9 binding in all target sites tested (Fig. 1, pink bars). Together, these data show that changes in the cellular environment directly influence SOX9 binding to its target sites.

2.2. Measuring SOX9 activity by FRAP

The aim of our investigation was to apply FRAP to directly observe changes in SOX9 transcriptional activity at short time scales, in the presence of external stimuli, in a small number of cells. Most of the DNA binding proteins show biphasic behavior, and the FRAP recovery curve for transcription factor binding and mobility is ideally fitted using a diffusion-uncoupled two-component fit (Eq. (2)). Presence of a shoulder in the FRAP curve indicates that at least two binding reactions contribute to the FRAP recovery, namely, 'fast' and 'slow' diffusion corresponding to 'weak' and 'strong' binding interactions. The fast diffusing population constitute non-specific and weak DNA binding SOX9-mGFP, whereas the slow diffusing population of SOX9-mGFP transiently

interacts and exchanges at the binding sites in DNA (i.e. the binding reactions), leading to slow mobility. [25,26].

DNA binding proteins, such as transcription factors, move randomly inside the nucleus and bind to the DNA at random intervals. On average, proteins reside on DNA for a period of time (called the mean residence time) and dissociate and move to another binding site. Thus, the mobility of DNA binding proteins is determined by two factors: namely, the translational mobility between binding sites (fast diffusion, non-specific and weak DNA binding,) and the binding reactions (slow diffusion, specific and strong DNA binding,). SOX9-mGFP FRAP recovery curves were more accurately fit by two exponential fits (Fig. S1. A, B), indicating there are at least two populations contributing to FRAP recovery, as shown for other proteins [26,27]. Further, to ensure that the SOX9-mGFP FRAP recovery is diffusion-uncoupled, we did two simple tests. Both tests are based on the principle that diffusion is an extremely fast event and does not depend on the spatial scale. First, we did FRAP measurements with different bleach ROI size (2.9 μm, 4.7 μm, and 6.4 μm diameter) and observed almost no change in the FRAP curve (Fig. S1. A) [25,28]. Second, we measured the FRAP recovery at different locations of the bleach spot and found almost no change in the FRAP curve (Fig. S1. B) [25,27]. These two tests indicate that the SOX9-mGFP recovery is diffusion-uncoupled.

Explanation of FRAP parameters: The ratio $\boldsymbol{A_1/A_2}$ refers to the increase or the decrease of fluorescent moieties in the A_1 compared to A_2 populations (i.e. the ratio of the fast diffusing population to the slow diffusing population). Recovery half-time ($t_{½}$) of A_1 and A_2 refers to 50% of time required to achieve full recovery of fast and slow diffusing populations respectively. Speed of the recovery indicates the strength of the binding - higher the speed, lower the binding strength and vice versa [25]. In contrast, mGFP has no binding sites inside the cell and serves as a good internal control for unbound protein, as it very quickly recovers to 100% after photobleaching (Fig. S2 and Table 1).

2.3. BMP-7 decreases SOX9 mobility

To investigate whether the increased DNA binding of SOX9 to its target sites correlated to a decrease in mobility, we performed FRAP on C-20/A4 cells that were transiently transfected with SOX9-mGFP. Cells were treated with BMP7 (100 ng/ml) for 1 h and the changes in mobility such as the immobile fraction, the ratio of the fast diffusing population to the slow diffusing population and $t_{½}$ of the SOX9-mGFP was compared to the untreated control. In the presence of BMP7, the SOX9-mGFP immobile fraction (IF) increased significantly from 53.6% to 65.7% (Fig. 2). The increase in IF was accompanied by significantly longer $t_{½}$ for both slow and fast diffusing populations of SOX9-mGFP. Ratio of the fast diffusing population is significantly decreased compared to the untreated control (Fig. 2 and Table 1), indicating that a higher fraction of the SOX9 population is bound to DNA leading to increased SOX9 activity. This corresponds to the results of the ChIP assay.

2.4. WNT3a increased SOX9 mobility

We examined the role of WNT3a in regulating SOX9 mobility by FRAP. Treatment of C-20/A4 cells with 10 ng/ml of recombinant WNT3a for 20 min significantly reduced the SOX9-mGFP immobile fraction by 13% (Fig. 3, blue boxes and Table 1). Recovery half-time of the fast diffusing population of SOX9-mGFP changed significantly as compared to the control. However, neither the $t_{½}$ of the slow diffusing population nor the ratio of the fast diffusing population changed significantly, compared to the untreated control (Fig. 3 and Table 1). There was a significant difference of WNT3a treatment for all parameters as compared to the BMP7 treatment. This indicates that in the presence of WNT3a more SOX9-mGFP is destabilized from DNA, rendering the protein more mobile. However, an increased concentration of WNT3a (200 ng/ml) did not further increase the SOX9-mGFP mobile fraction (Supplementary Fig. S3).

2.5. IL1β increased SOX9 mobility, which was reversed by iNOS inhibition

We have not found any reports indicating that IL1β has an inhibitory role in SOX9 protein activity, however, we have previously found that IL1β exposure decreased expression of the SOX9 target genes *COL2A1* and *ACAN* [29]. We hypothesized that IL1β decreases *ACAN* and *COL2A1* expression by inhibiting SOX9 transcriptional activity and reducing SOX9 binding to DNA. SOX9-mGFP transfected C-20/A4 cells were treated with 10 ng/ml of recombinant IL1β for 20 min. This resulted in a decrease of the immobile fraction by 15%, which was similar to that found for WNT3a treatment (Fig. 3, pink boxes, and Table 1). There was no significant difference in the recovery half-time of the slow and the fast diffusing populations compared to the untreated control. The ratio of the fast and slow diffusing populations did not change significantly as compared to control cells (Fig. 3 and Table 1). Changes between BMP7 and IL1β treated conditions are significant for all parameters.

IL1β, and other pro-inflammatory cytokines, can activate iNOS (inducible Nitric Oxide Synthase) that stimulates nitric oxide (NO) production [30,31]. It has been shown that in the presence of NO, the transcription factor NFκB binds more tightly to its target gene, thereby increasing the level of target gene expression. The role of NO in OA is well documented [32–34]. Animal experiments have shown that inhibiting iNOS results in a decrease in the expression of catabolic factors [35].

To investigate if iNOS/NO plays a role in the decrease of SOX9 activity, we treated the mGFP-SOX9 transfected cells with a combination of IL1β and 1400 W, a potent iNOS inhibitor. We first treated C-20/A4 cells with IL1β for 60 min, and then added 1400 W (100 μM/ml) and measured the SOX9-mGFP mobility after 20 min by FRAP. In the presence of both IL1β and 1400 W, the SOX9-mGFP immobile fraction was restored to the level of the untreated control. This data suggests that IL1β signaling regulates SOX9 activity through iNOS. (Fig. 4, green boxes and Table 1). Adding 1400 W in the presence of IL1β increased the $t_{½}$ of both slow and fast diffusing populations, while the ratio did not change significantly compared to the untreated control. (Fig. 3, green boxes and Table 1). Indeed, the $t_{½}$ more closely resembled the $t_{½}$ of SOX9-mGFP in the presence of BMP7. Treatment with 1400 W alone slightly increased SOX9-mGFP mobility, but not to the level of IL1β treatment. However, changes between 1400 W and IL1β + 1400 W are not significant (Supplementary Fig. S4. A and B, Table S1). In addition, FRAP was performed after 4 h of either IL1β or IL1β with 1400 W treatment (Supplementary Fig. S5). There were no significant differences between 20 min and 4 h treatment.

2.6. Mutant variants of SOX9-mGFP that are DNA binding impaired, do not respond to BMP7 treatment

To ensure that our SOX9-mGFP is functional, we generated two non-functional SOX9-mGFP variants by site directed mutagenesis. SOX9 can form dimers with other SOX9 proteins, as well as with SOX5/6 to regulated target gene expression [36]. To investigate the role of protein dimerization in the DNA binding and response to external stimuli, we generated a mutation of SOX9 (A76E), which is a non-dimerization mutant [37]. To prevent SOX9 DNA binding, we generated SOX9 (W143R), a non-DNA binding mutant [38]. SOX9 (A76E) weakly binds to DNA as compared to wild-type (*wt*) SOX9-mGFP as evidenced by decreased DNA binding localization pattern and a slightly lower protein mobility as compared to the SOX9(W143R) mutant. SOX9(W143R) did not bind to DNA as evidenced by quick recovery after photo-bleaching, the absence of a DNA binding localization pattern and near zero immobile fraction (Fig. 4. A, B, C). With the exception of differences in the recovery half-time of fast diffusing populations, there was no significant difference in FRAP recovery rates between the SOX9(A76E) and

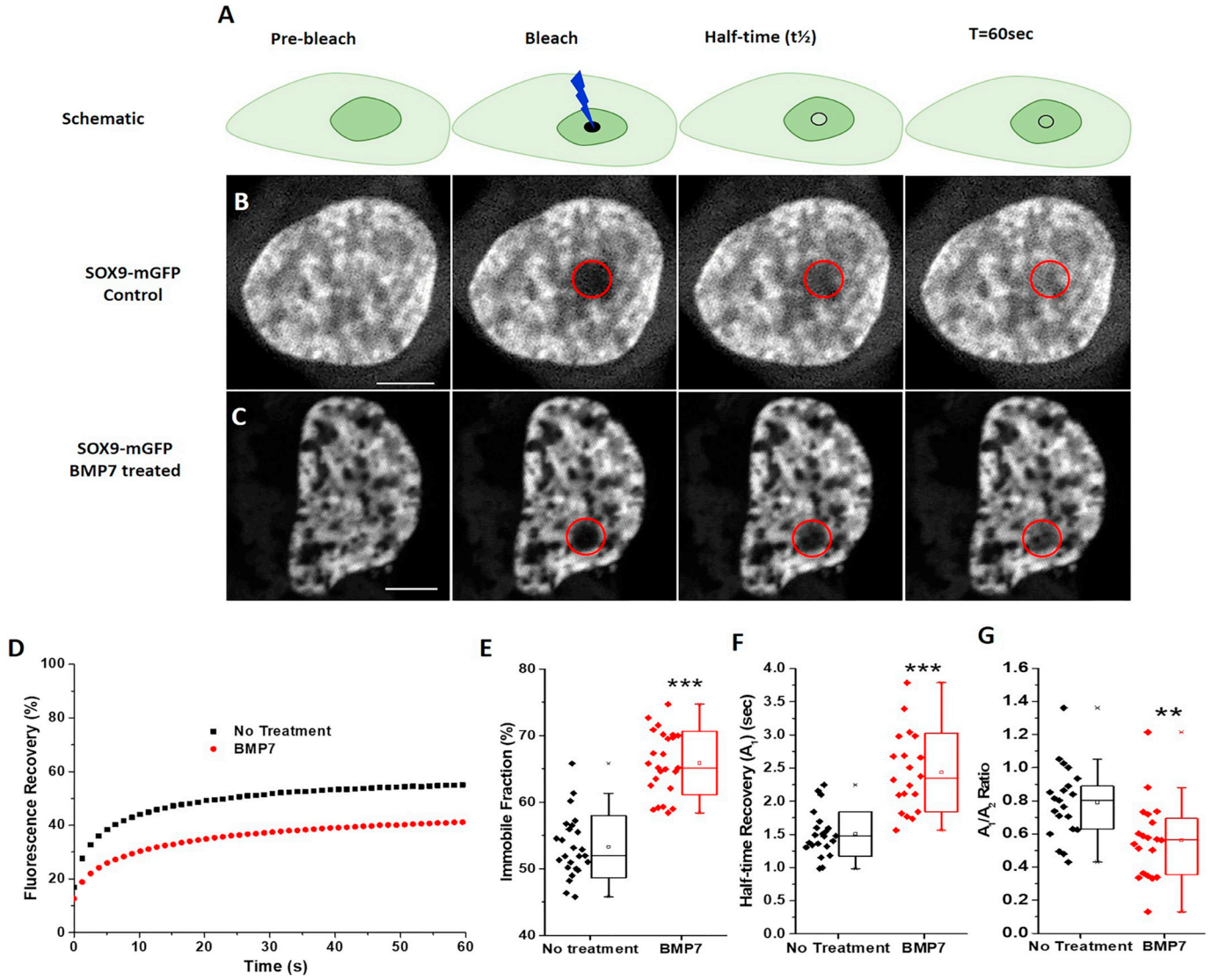

Fig. 2. BMP7 increases SOX9 activity and this can be observed by FRAP. A. Schematic diagram illustrating the FRAP procedure. A small circular region of a nucleus expressing fluorescent protein is photo-bleached with high-intensity laser and recovery of the fluorescence at the bleached region is monitored over the period. B. Fluorescence recovery of SOX9-mGFP after photobleaching without any treatment. C. Fluorescence recovery of SOX9-mGFP is less after BMP7 treatment compared to the untreated control. Bar size: 5 µm. D. Averaged ($n \geq 20$) FRAP curves show that the mobility of SOX9-mGFP is less after BMP7 treatment as compared to the untreated control. E. SOX9-mGFP binding to DNA is increased in the presence of BMP7 Higher the fluorescence recovery, lesser the immobile fraction. F. BMP7 treatment prolongs recovery half-time of slow diffusing population of SOX9-mGFP indicating slower mobility compared to the control. G. BMP7 treatment decreased the ratio of fast diffusing population compared to control, indicating that more SOX9-mGFP is bound to DNA. The individual measurements are indicated on the left of each boxplot. The boxplots indicate 95% CI. Mann-Whitney *U* test was used for statistical analysis. Statistical significance was calculated between the untreated control and the treatments and between the treatments as stated. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Table 1
Calculated FRAP rates. Immobile fraction (IF), recovery half-time ($t_{½}$), and ratio of the slow (A_1) diffusing population to the fast (A_2) diffusing population of the SOX9-mGFP in response to external stimuli.

Treatment	IF (%)	$t_{½}$ of A_1 (s)	$t_{½}$ of A_2 (s)	Ratio A_1/A_2
No treatment	53.6 ± 4.8	1.51 ± 0.33	13.79 ± 5.26	0.82 ± 0.26
BMP7	65.7 ± 4.9	2.43 ± 0.59	19.46 ± 5.36	0.56 ± 0.23
WNT3a	39.6 ± 7.2	1.78 ± 0.39	15.42 ± 5.21	0.94 ± 0.26
IL1β	38.0 ± 7.4	1.64 ± 0.53	14.62 ± 5.29	0.91 ± 0.24
IL1β + 1400 W	47.5 ± 7.6	2.18 ± 0.56	17.43 ± 3.58	0.79 ± 0.22
1400 W	46.2 ± 5.4	1.78 ± 0.45	15.94 ± 4.27	0.84 ± 0.26
Mobility of mGFP	1.2 ± 5.3	0.78 ± 0.24	–	–

± = standard deviation.

SOX9(W143R) mutants. However, the FRAP rates are significantly different from the *wt* SOX9-mGFP. To test whether BMP7 could increase DNA binding of SOX9 (A76E) and W143R, we treated SOX9 (A76E) or W143R expressing C-20/A4 cells with BMP7 (100 ng/ml) for 1 h. Our FRAP rates show that BMP7 had little effect on DNA binding properties of A76E and W143R (Fig. 4. B, C, D, E, F and Table 2).

Further, the amplitude of the fast diffusing populations of the mutated SOX9-mGFPs are almost two-fold higher than the *wt* SOX9-mGFP (Fig. 4. F and Table 2), indicating that most of the mutated SOX9-mGFP is weakly bound to DNA. SOX9 (A76E) and SOX9 (W143R) show decreased or no DNA binding and thus no slow-moving population (A_2) is expected to be present in these FRAP measurements. However, the single component fit did not fit properly (data not shown) with the FRAP curves of these mutants and the observed A_2 population (Table 2) could be due to their non-specific binding with DNA and complexing with other proteins which increase their molecular weight and thereby

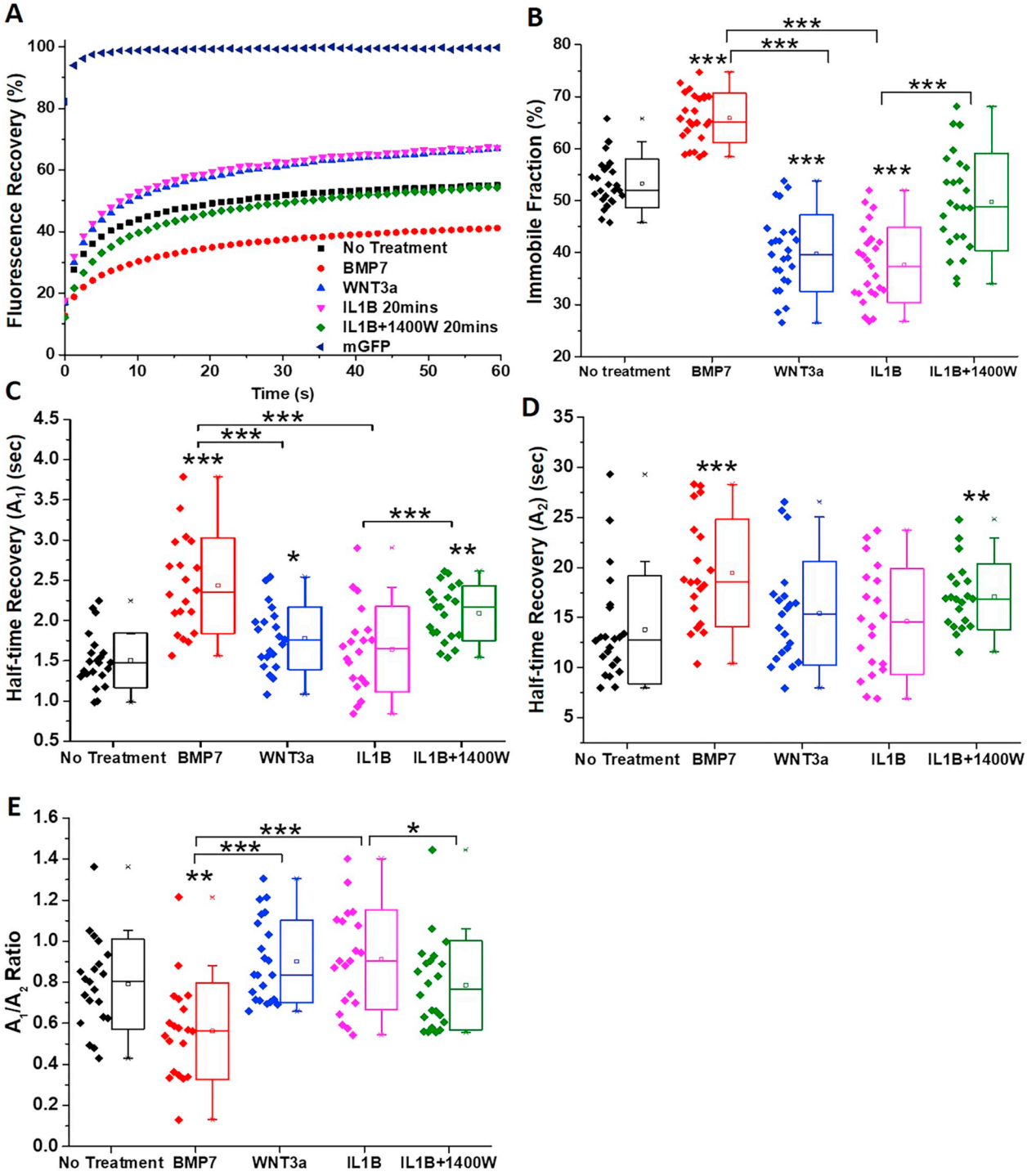

Fig. 3. Changes in SOX9-mGFP mobility in response to external stimuli measured by FRAP. BMP7 (red) decreased SOX9-mGFP mobility, while WNT3a (blue) and IL1β (pink) increased the mobility. Addition of iNOS inhibitor 1400 W in the presence of IL1β (green) restored the SOX9-mGFP mobility to the control levels. (A) Averaged ($n \geq 20$) fluorescence recovery curves of SOX9-mGFP in response to external stimulation. (B) WNT3a and IL1β decreased the immobile fraction of SOX9-mGFP. In the presence of IL1β, 1400 restored SOX9-mGFP mobility to the control level. (C, D) Recovery half-time of the fast diffusing (A_1) and the slow diffusing (A_2) population of SOX9-mGFP, respectively. (E) WNT3a and IL1β treatments increased the A_1 fraction of the SOX9-mGFP. The individual measurements are indicated on the left of each boxplot. Boxplots are displayed as 95% confidence interval with standard deviation. Mann-Whitney *U* test was used for statistical analysis. Statistical significance was calculated between the untreated control and the treatments and between the treatments as stated. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

the mobility is obstructed. The recovery half-time of A_2 of both the SOX9 (A76E) and SOX9(W143R) is significantly decreased as compared to *wt* SOX9-mGFP (Fig. 4. E and Table 2). This implies that their binding strength is less, resulting in a lower residence time on DNA.

2.7. FRAP readings correlate with long-term gene expression levels

To evaluate whether the immediate effects of BMP7, WNT3a and IL1β on SOX9-mGFP mobility and DNA binding correlate to transcription of SOX9 target genes in the long term, we measured the target gene expression levels at 24 h after treatment. For each ligand, we tested a

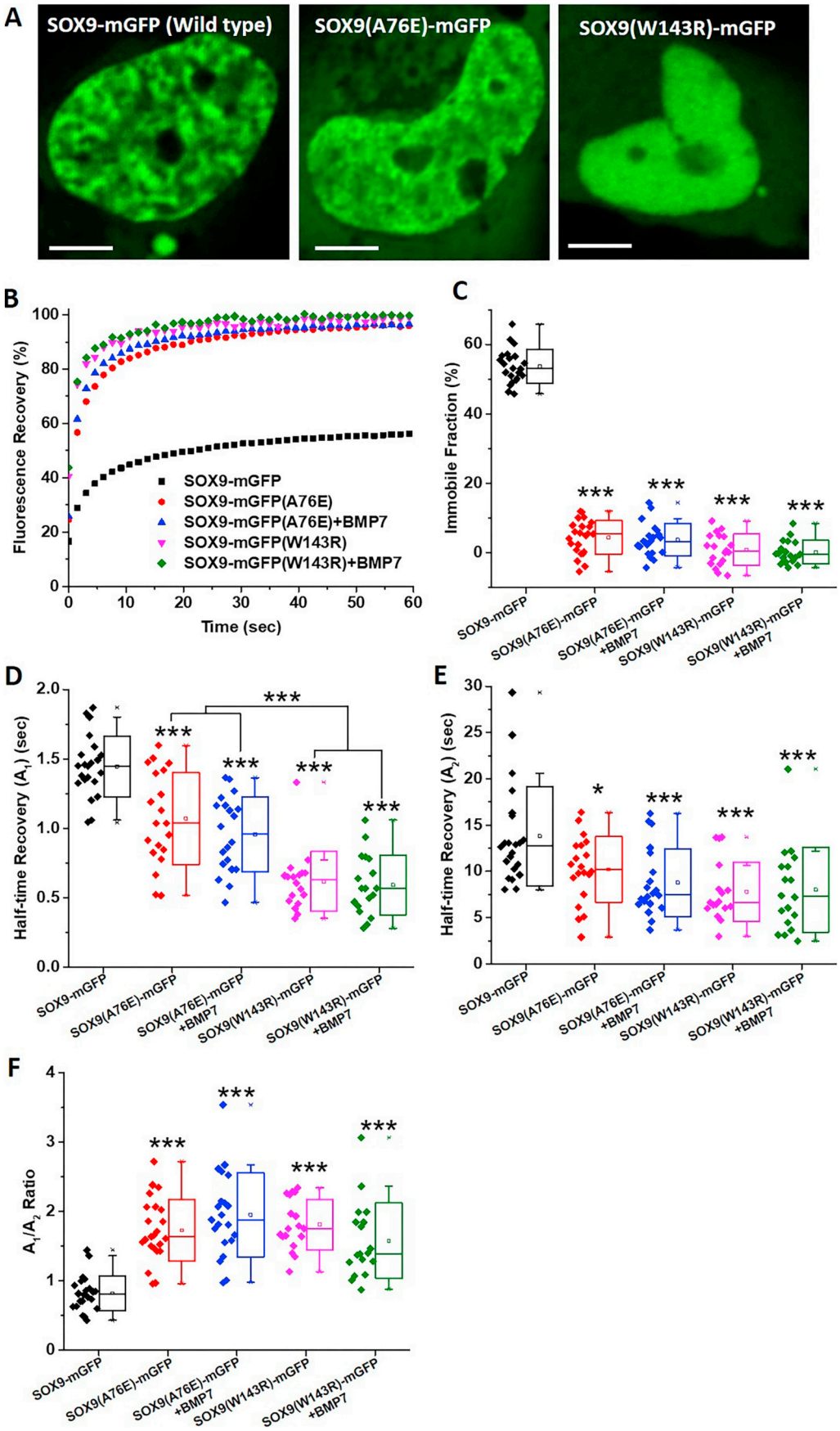

Fig. 4. Mutant SOX9-mGFPs did not respond to BMP7 treatment. (A) Nuclear localization pattern of wt and mutant SOX9-mGFP. Highly patterned nuclear localization was observed in the wt SOX9-mGFP. Non-dimerizing mutant SOX9 (A76E)-mGFP showed less patterned and more diffused localization pattern in the nucleus as compared to wt SOX9-mGFP. Non-DNA binding mutant SOX9(W143R)-mGFP showed highly diffused nuclear localization pattern as compared to wt SOX9-mGFP. (B) Averaged FRAP recovery curves ($n \geq 20$) show both the SOX9-mGFP mutants fully recover after photobleaching and results in near zero immobile fraction (C). (D) Recovery half-time of fast diffusing population of both the mutant SOX9-mGFPs is significantly decreased as compared to wt SOX9-mGFP. However, recovery half-time of SOX9(A76E) -mGFP is slightly higher than SOX9(W143R)-mGFP due to its weak DNA binding. (E) Recovery half-time of slow diffusing fraction of both the SOX9-mGFP mutants are significantly lower than the wt SOX9-mGFP. (F) Fast diffusing population of SOX9-mGFP is significantly higher in both the SOX9-mGFP mutants as compared to the wt SOX9-mGFP. BMP7 (100 ng/ml) treatment did not increase the DNA binding of mutated SOX9-mGFPs. The individual measurements are indicated on the left of each boxplot. Boxplots are displayed as 95% confidence interval with standard deviation. Scale bar: 5 μm. Mann-Whitney *U* test was used for statistical analysis. Statistical significance was calculated between the wt and mutated SOX9-mGFP and between the mutants as stated. $*p < 0.05$, $***p < 0.001$.

Table 2
Calculated FRAP rates of mutant SOX9-mGFPs. Immobile fraction (IF), recovery half-time ($t_{½}$), and ratio of the slow (A_1) diffusing population to the fast (A_2) diffusing population of the SOX9-mGFP in response to external stimuli.

SOX9-mGFP and treatment	IF (%)	$t_{½}$ of A_1 (s)	$t_{½}$ of A_2 (s)	Ratio A_1/A_2
SOX9-mGFP	53.6 ± 4.8	1.51 ± 0.33	13.79 ± 5.26	0.82 ± 0.26
SOX9(A76E))-mGFP	4.2 ± 1.6	1.2 ± 0.42	10.2 ± 3.6	1.70 ± 0.45
SOX9(A76E)-mGFP + BMP7	4.0 ± 4.4	0.95 ± 0.27	8.8 ± 3.6	1.96 ± 0.62
SOX9(-W143R)-mGFP	0.5 ± 4.4	0.62 ± 0.21	8.5 ± 4.4	1.81 ± 0.36
SOX9(W143R)-mGFP + BMP7	0.2 ± 3.4	0.59 ± 0.22	8.0 ± 4.6	1.58 ± 0.54

± = standard deviation.

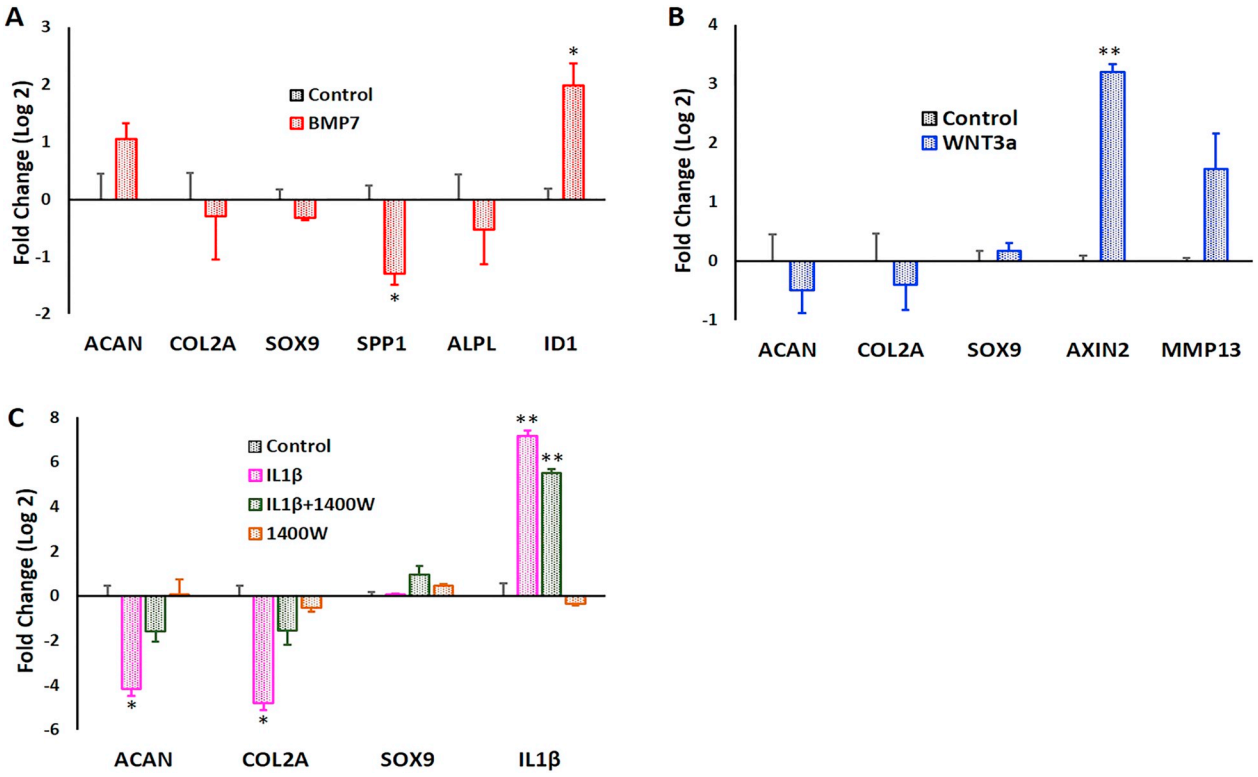

Fig. 5. Gene expression levels quantified by qPCR after 24 h of BMP7, WNT3a, IL1β or IL1β + 1400 W treatment. (A) BMP7 upregulated ACAN, while downregulating COL2A. (B) WNT3a downregulated SOX9 target genes ACAN, COL2A and upregulated the WNT target gene AXIN2. (C) IL1β upregulated IL1β and downregulated SOX9 target genes ACAN and COL2A. iNOS inhibitor 1400 W, in the presence of IL1β, partially reversed the IL1β induced effect. Unpaired, 2-tailed, Student *t*-test was performed between control and treatments. $*p < 0.05$, $**p < 0.01$.

known target gene of its canonical pathway, as well as the SOX9 cartilage-specific target genes *COL2A1* and *ACAN*.

BMP7 induced the expression of *ACAN* but not *COL2A1*. The expression of the BMP7 target gene *ID1* increased in the presence of BMP7, while the hypertrophic markers *SPP1* and *ALPL* were downregulated as compared to the control (Fig. 5. A, red bars). The expression *ACAN* and *COL2A1* were downregulated in the presence of WNT3a, while the WNT target *AXIN2* was upregulated. The hypertrophic marker gene *MMP13* [24] was upregulated in the presence of WNT3a (Fig. 5. B, blue bars).

As expected, the presence of IL1β upregulated expression of the targets *IL1β*, while *ACAN* and *COL2A1*, were downregulated (Fig. 5. C, pink bars). Addition of 1400 W in the presence of IL1β restored SOX9 activity as evidenced by the upregulation of *COL2A1* and *ACAN*. However, treatment with 1400 W alone did not influence gene expression (Fig. 5. C, brown bars).

To investigate the influence of SOX9-mGFP overexpression on its activity, we also quantified gene expression levels in response to BMP7, WNT3a and IL1β in C-20/A4 cells transfected with SOX9-mGFP. With slight differences that are possibly due to the overexpression of SOX9, these levels correlate with the gene expression patterns of non-transfected cells (see Supplementary Fig. S6. A, B, C).

3. Discussion

We have demonstrated that FRAP may be used as a fast and reliable method to study SOX9 transcriptional activity on short time scales. We show that the activity of SOX9 transcription factor is linked to DNA binding. Moreover, our data show that a reduction or an increase in transcriptional factor mobility as measured by FRAP correlates to DNA binding and transcriptional activity as evidenced by ChIP and qPCR results, respectively. FRAP directly measures DNA binding and mobility of the transcription factors and this can be used as an indicator of transcriptional activity.

SOX9 is the master transcription factor in chondrocyte development and plays a central role in cartilage homeostasis. We used a number of factors to map their influence on SOX9 protein activity. While BMP7 was shown to enhance SOX9 activity, WNT3a and IL1β inhibited SOX9 activity. This is the first time that these effects are shown at the level of SOX9 protein activity. There are many reports have studied the role of WNT/β-catenin and IL1β signaling in SOX9 transcriptional activity by measuring SOX9 promotor activity (by luciferase assay) or mRNA or

protein expression of SOX9 and/or its target genes after several hours of stimulation. However, none of these studies has directly investigated SOX9 protein activity in live cells in response to external stimulation due to unavailability of suitable methods. To our knowledge, this is the first report that the upstream signals have direct effect on SOX9 transcriptional activity on very short time scales (20 min to 1 h after treatment), that results in changes in longtime target gene expression levels [18,24,39,4]. Further, mapping of upstream factors regulating SOX9 activity in human primary chondrocytes (hPCs) is necessary to understand chondrocyte homeostasis and OA pathophysiology.

In studying SOX9 transcriptional activity by FRAP, we look for a percentage of change in immobile fractions in addition to change in the $t_{½}$ of A_1 and A_2 and A_1/A_2 of the transcription factor. In a cellular milieu, the term "effective diffusion" indicates the recovery that mimics diffusion, but at a rate that is slowed by binding interactions [25] with other proteins and co-factors. One needs to take these latter factors into account only if they investigate protein diffusion kinetics. Another important factor would be translocation of the protein of interest from cytoplasm to nucleus or vice versa. Nuclear import happens at a time scale of minutes [41], thus this is especially important for FRAP studies with a duration from several minutes to hours [42,43]. Cytoplasmic levels of SOX9-mGFP in C20/A4 cells were very less (< 10%), compared to its nuclear localization level. Thus it is highly unlikely that the nuclear import of SOX9-mGFP contributes to FRAP measurements with very short duration (60 s).

We observed a significant decrease of SOX9 activity in the presence of WNT3a and IL1β, whereas, SOX9 activity was increased in the presence of BMP7 as evidenced by the decrease or increase in the immobile fraction. The ratio of the fast diffusing population versus the slow diffusing population (A_1/A_2) showed that the BMP7 treatment increased the slow diffusing SOX9-mGFP population, indicating more SOX9 molecules were bound to DNA. Whereas, WNT3a and IL1β treatments have increased the unbound population, indicating less SOX9 was bound to DNA, as compared to the untreated control.

WNT3a and IL1β treatments destabilized SOX9-mGFP from DNA and as expected, we found a higher mobility and thus lower immobile fractions, but longer $t_{½}$ rates of SOX9-mGFP. However, the recovery kinetics of the transcription factor are highly dependent on the interactions with other transcription factors and co-factors following stimulation [25,44]. In effect, higher (or lower) mobile fractions are not always associated with a shorter or longer $t_{½}$. Significant differences in $t_{½}$ between the treated conditions and/or the untreated conditions of the fast and the slow diffusing populations indicate the possible changes in intermolecular interactions with different treatments. For example, in the presence of IL1β, the iNOS inhibitor 1400 W restored the SOX9-mGFP activity to the control levels, indicating that IL1β inhibits SOX9 transcriptional activity through the iNOS pathway. However, blocking iNOS by 1400 W resulted in a significantly slower recovery of both the fast and the slow diffusing populations, as compared to the control or the IL1β treatments. This indicates a possible differential intermolecular interaction, leading to an increase in the molecular mass of SOX9-mGFP, resulting in a slower recovery.

Destabilization of a transcription factor from DNA is a faster event compared to the binding to DNA as binding involves several steps, including gaining access to the binding sites, chromatin remodeling and priming of enhancer sites [45]. BMP7 may also promote the complex formation of SOX9 with other proteins, contributing to the slower recovery of both the fractions. That can explain why we could not observe changes in SOX9-mGFP mobility within 20 min after BMP7 treatment (data not shown). Increasing the treatment time to 60 min allowed us to see changes in the mobility, possibly because the binding process involves multiple events.

Transcription factor binding to DNA does not always result in target gene expression. Transcription factors can repress transcription or necessary co-factors are not bound to the transcription factor [46]. However, transcription factor binding to DNA is paramount for its transcriptional regulation. In the presence of external stimuli, the change in SOX9 immobile fraction is within 15% as compared to the control. This indicates that although a change in immobile fraction alters gene expression, there are SOX9 binding sites that are not affected by external factors. A possible reason could be that at these binding sites SOX9 activity is regulated by a different mechanism, such as binding of a co-factor or a repressor protein.

Binding of the transcription factors to DNA at their target sites that leads to gene transcription or repression is termed functional binding, whereas binding to off target sites is called non-specific binding. Previous studies of transcription factors by FRAP claim that the FRAP predominantly measured either specific or non-specific binding or both [47]. In our studies, changes in SOX9 mobility (i.e. immobile fraction, and the ratio of fast and slow-moving SOX protein, A_1/A_2) measured by FRAP directly influenced transcription, which correlated with both DNA binding and target gene expression levels. The combined qPCR and ChIP data indicate that our FRAP measurements predominantly correlated with the functional binding of SOX9, although there is a considerable contribution from non-specific binding and unbound population to the fluorescence recovery. If non-specific binding dominated the FRAP measurements, changes in response to the stimulation would not have influenced the target gene expression levels. The traditional methods used to study the activity of SOX9 are neither fast nor direct and lack spatiotemporal resolution of gene and protein expression. The advantages of FRAP over traditional methods are: it is relatively simple, comparatively fast, less expensive and cells can be measured immediately (20 min to 1 h) after stimulation at the single cell resolution. This enables the capture of the immediate changes in the transcriptional activity following stimulation. This provides useful real-time dynamics of transcriptional activity in terms of immobile fraction, ratio of fast diffusing population to slow diffusing population, diffusion constants and $t_{½}$, which one cannot obtain by qPCR or western blotting. In addition, FRAP measures real-time kinetics of transcriptional activity at the single cell level. We have shown that the immediate changes in the activity of SOX9 protein in response to the external stimuli can be measured by FRAP with high spatiotemporal resolution.

While the traditional methods are mostly performed with endogenous expression of proteins. FRAP requires cells be overexpressing proteins tagged with a fluorescent protein. To ensure this overexpression does not influence our FRAP results, we performed qPCR experiments with and without SOX9 overexpression and observed that the gene expression patterns are identical. Our ChIP results confirm that the increase of SOX9-mGFP mobility in the presence of WNT3a and IL1β is due to destabilization of SOX9 from DNA, and qPCR results show that this effect results in a decrease in long-term gene expression levels. Further, in the presence of the SOX9 anabolic factor BMP7, the effects are reversed. In addition, overexpression of binding impaired, non-functional SOX9-mGFP mutants failed to respond to the external stimuli and their FRAP-rates are significantly different from functional wt SOX9-mGFP. This indicates that the wt SOX9-mGFP is functional and its overexpression did not influence our FRAP results.

Studying the transcriptional activity in cells of rare samples such as OA cartilage tissue is a challenging task, as the cells dedifferentiate during expansion. Studying the activity of a transcription factor before dedifferentiation is essential to properly map the cell type specific transcriptional regulation. Here, FRAP provides another opportunity that it requires only a few thousand cells (depending on plating area) for plating and less than hundred cells for FRAP measurements, enabling the study of transcriptional activity in rare samples that do not need expansion to obtain a high enough cell number. In addition, the method is performed at the single cell resolution, enabling observation of a spread in the response of single cells. This heterogeneity in cell responses cannot be studied with conventional methods.

SOX9 plays a pivotal role in development, cell differentiation, tissue/cell homeostasis and disease pathophysiology such as OA [1]. Mapping the factors that differentially regulate the dynamics and

activity of SOX9 in healthy and OA chondrocytes will shed new light on chondrocyte physiology and OA pathophysiology, thereby identifying possible therapeutic targets. Here, we have shown that the external stimulation by BMP7, WNT3a and IL1β can directly influence transcriptional activity by either increasing or decreasing the binding of SOX9 to DNA. These changes in DNA binding could be measured by FRAP, which has several advantages over traditional methods.

4. Materials and methods

4.1. Plasmids

The vector expressing SOX9-mGFP was constructed by cloning mGFP (PS100040, Origene) with the C-terminal of wild type SOX9 (RC208944, Origene) using *Sgf*I and *Mlu*I restriction sites. The correct reading frame of the fusion construct was verified by sequencing. Non-dimerizing and non-DNA binding mutated SOX9-mGFP, A76E and W143R respectively, were generated from SOX9-mGFP by site-directed mutagenesis (210518, Agilent). Primer sequences used for site directed mutagenesis are specified in Table S2. Mutations were verified by sequencing.

4.2. Cell culture and transfection

Immortalized human juvenile costal chondrocytes (C-20/A4 cells) [20] were cultured in DMEM (Invitrogen, USA) supplemented with 10% FBS (Sigma) without antibiotic at 37 °C with 5% CO_2. Cells were cultured on glass coverslips and transfected a day before FRAP measurements. Cells were plated in 24 well plate a day before transfection. Lipofectamine LTX with Plus Reagent (Invitrogen, USA) was used for transfection and the manufacturer's protocol was followed.

4.3. Imaging buffer

Imaging was performed in Tyrode's buffer with freshly added 20 mM glucose (GIBCO) and 0.1% BSA (Sigma) [48]. Tyrode's buffer is composed of 135 mM NaCl (Sigma), 10 mM KCl (Sigma), 0.4 mM $MgCl_2$ (Sigma), 1 mM $CaCl_2$ (Sigma), 10 mM HEPES (Acros organics), pH adjusted to 7.2, filter sterilized and stored at −20.

4.4. Cytokine/antagonist treatments

Cells were washed with Imaging buffer, and either WNT3a (R&D Systems, Minneapolis, USA) or IL1β (PeproTech, USA) was supplemented to the imaging buffer at a final concentration of 10 ng/ml, unless otherwise noted. FRAP was performed starting from 20 min after treatment, unless otherwise noted. The iNOS inhibitor 1400 W (Cayman chemical, Michigan, USA) was added to the IL1β-treated cells after 20 min, at a final concentration of 100 μg/ml while maintaining the IL1β concentration. FRAP was performed after 20 min of 1400 W addition. For BMP7 treatment, cells were incubated in 100 ng/ml of BMP7 (R&D Systems, Minneapolis, USA) for 1 h.

4.5. Fluorescence Recovery After Photobleaching (FRAP)

Transiently transfected C-20/A4 cells grown on glass coverslips were maintained in imaging buffer with or without external factors. The FRAP measurement was performed in Nikon A1 laser scanning confocal microscope with 60×/1.2 NA water immersion objective, 488 nm Argon laser at 0.5% laser power during imaging and 1 iteration of 50% laser power during photo-bleaching (Nikon, Japan). The temperature was maintained at 37 °C with an OkaLab temperature controller. For FRAP, a frame size of 256 × 256 pixels covering the whole nucleus was scanned at 4 frames/s for 60 s post-bleach. The pixel size was 0.12 μm. A representative circular region of 2.9 μm diameter was bleached with one iteration (60 ms) of high intensity laser (50%). Twenty-five pre-bleach images were taken and the last 10 pre-bleach fluorescence intensity values were averaged to normalize the post-bleach fluorescence recovery curve. We performed FRAP experiments on at least 20 cells per condition and repeated experiments at least twice. Individual FRAP measurement curves were averaged to get a single FRAP curve. To assess the statistical significance between the conditions Mann-Whitney *U* tests were applied using Origin software. Matlab™ was used to analyze the FRAP data and the script is available upon request. The difference in terminologies used in the manuscript and in the Matlab script is tabulated in the supplementary information (see Supplementary, Table S3).

4.6. Formulae used in our FRAP calculations

We used a previously described diffusion uncoupled, two-component method [26] and applied it to analyze wild-type and mutated SOX9-mGFP FRAP data. A single-component fit was used to analyze mGFP mobility.

$$\text{Single component fit: } F(t) = y0 + A_1(1 - e^{-t/\tau}) \quad (1)$$

where F_0 is the value of the fluorescent intensity at the first post-bleach frame, A_1 is the amplitude of the fast diffusing population and τ is the time constant.

$$\text{Two} - \text{component fit: } F(t) = y0 + A_1(1 - e^{-t/\tau 1}) + A_2(1 - e^{-t/\tau 2}) \quad (2)$$

where A_2 is the amplitude of slow diffusing population, τ_1 and τ_2 are the time constants of A_1 and A_2 respectively.

$$\text{Half} - \text{time to recover: } t_{1/2} = \ln(2) * \tau \quad (3)$$

$$\text{Immobile fraction: } IF = F_I - F_E \quad (4)$$

where F_I is the initial intensity and F_E is the end value of the recovered intensity.

We determined the effective diffusion constant for mGFP that shows a single component behavior. However, the initial diffusion after bleaching was faster than we could measure. Therefore, the post-bleach intensity and thus the effective diffusion constant were underestimated. To correct this, we determined the post bleaching intensity with GFP in fixed cells (on average, post-bleach $I = 20\%$ of pre-bleach I) and used this as an initial value in our fits.

4.7. Chromatin immunoprecipitation (ChIP)

Cells were cultured in 10 cm plates, transfected with SOX9-mGFP and treated with WNT3a (10 ng/ml), BMP7 (100 ng/ml) and IL1β (10 ng/ml) for 1 h in serum free media. ChIP was carried out using an EZ-ChIP kit (Millipore, USA, Cat# 17-371), according to the manufacturer's guidelines. A ChIP validated SOX9 antibody [37] (AB5535, EMD Millipore) was used at 1:500 dilution to pull out SOX9-DNA complexes. ChIP experiments ware performed in duplicates with two experimental repetitions. The immunoprecipitated chromatin was analyzed for SOX9 binding regions ACAN enhancer, COL2A Intron-1, COL2A Intron-6 and COL2A promoter regions by RT-qPCR [21,49,50]. C_t values of control and treated conditions were normalized to the corresponding input values. Primer sequences are specified in the Supplementary information (Table S2).

4.8. RT-qPCR

C-20/A4 cells (with and without overexpressing SOX9-mGFP) cultured in 24-well plates were treated with WNT3a (100 ng/ml) or BMP7 (100 ng/ml) or IL1β (10 ng/ml) or IL1β (10 ng/ml) + 1400 W (100 μM) for 24 h. mRNA was isolated using NucleoSpin RNA II kit (Macherey-Nagel). Purity and concentration of RNA samples were measured by Nanodrop 2000. cDNA was synthesized from total RNA with iScript cDNA synthesis kit (Bio-Rad). Real-time PCR analysis was carried out using SYBR Green mix (Bioline) in Bio-Rad CFX-100 RT-PCR. Gene

expression is reported as the relative fold-change ($\Delta\Delta C_t$) [51] and is normalized to untreated control. Primer sequences are specified in the Supplementary information (Table S2).

Transparency document

The Transparency document associated with this article can be found, in online version.

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Author contributions

KG: designing and carrying out experiments, data collection and analysis, manuscript drafting, JH: helped with Matlab script for FRAP calculations, DL: provided initial Matlab scripts for FRAP analysis, JNP conceived idea to measure protein mobility in response to external factors, and derived hypotheses, KG, DL, MK and JNP: experimental design and data interpretation. All authors contributed to the writing of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbagrm.2018.11.001.

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