Elucidation of the functional roles of the Q and I motifs in the human chromatin-remodeling enzyme BRG1

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The Snf2 proteins, comprising 53 different enzymes in humans, belong to the SF2 family. Many Snf2 enzymes possess chromatin-remodeling activity, requiring a functional ATPase domain consisting of conserved motifs named Q and I–VII. These motifs form two recA-like domains, creating an ATP-binding pocket. Little is known about the function of the conserved motifs in chromatin-remodeling enzymes. Here, we characterized the function of the Q and I (Walker I) motifs in hBRG1 (SMARCA4). The motifs are in close proximity to the bound ATP, suggesting a role in nucleotide binding and/or hydrolysis. Unexpectedly, when substituting the conserved residues Gln758 (Q motif) or Lys785 (I motif) of both motifs, all variants still bound ATP and exhibited basal ATPase activity similar to that of wildtype BRG1 (wtBRG1). However, all mutants lost the nucleosome-dependent stimulation of the ATPase domain. Their chromatin-remodeling rates were impaired accordingly, but nucleosome binding was retained and still comparable with that of wtBRG1. Interestingly, a cancer-relevant substitution, L754F (Q motif), displayed defects similar to the Gln758 variant(s), arguing for a comparable loss of function. Because we excluded a mutual interference of ATP and nucleosome binding, we postulate that both motifs stimulate the ATPase and chromatin-remodeling activities upon binding of BRG1 to nucleosomes, probably via allosteric mechanisms. Furthermore, mutations of both motifs similarly affect the enzymatic functionality of BRG1 in vitro and in living cells. Of note, in BRG1-deficient H1299 cells, exogenously expressed wtBRG1, but not BRG1 Q758A and BRG1 K785R, exhibited a tumor suppressor–like function.

The eukaryotic DNA is densely packaged into chromatin with the nucleosome being the basal packaging unit. Approximately 147 bp of DNA are wrapped around an octamer of histones, forming the nucleosome core (1, 2). The association of histones with DNA and the further compaction of nucleosomal arrays into higher order structures of chromatin (3–5) inhibit the sequence specific binding of regulatory proteins to DNA. Chromatin-modifying enzymes have evolved to change the structure of chromatin and to regulate the accessibility to genomic DNA (6–8). Among them are ATP-dependent chromatin-remodeling enzymes, which move, evict, or rearrange nucleosomes (2). These highly abundant enzymes are required for the regulation of all DNA-dependent processes and usually act in the context of huge protein complexes, comprising up to 10 subunits or more (2).

Chromatin-remodeling enzymes belong to the Snf2 family of SF2 helicases, which is further subdivided in 24 subfamilies, comprising 53 enzymes in humans (9). Snf2 enzymes possess a conserved helicase region of high sequence similarity to Saccharomyces cerevisiae Snf2p (9). The helicase core is formed by two “recA-like” domains, being connected via a “linker” and forming a binding pocket for ATP (9, 10). The recA1 domain comprises motifs I, Ia, II, and III and the Q motif (N terminus of motif I), whereas the recA2 domain contains motifs IV to VI (9). Other well-conserved blocks are distributed over both domains as well as the linker region (9).

To date, little is known about the function of these conserved motifs in chromatin-remodeling enzymes. Motif V in the yeast Swi2(p)/Snf2(p) ATPase seems to couple the hydrolysis of ATP to the mechanism of chromatin remodeling of the ySWI2–SNF2 complex (Snf2 subfamily) (9), by contributing to nucleosomal substrate recognition (11). Point mutations in motif IVb (K1088A), motif VI (R1196K), and motif III (W935A) caused phenotypes in vivo, correlating in severity with the reduced ATP hydrolysis rates of the respective yeast SWI2–SNF2 complex mutants (IVb, 0%; VI, 3%; and III, 80% of the WT activity) (11, 12). Crystal structures of SF2 helicases (13) indicate that residues of the Q motif form hydrogen bonds with Nε7 of the adenine and that residues of the I motif contact the phosphates of the ATP (13, 10, 14). According to recent studies, a conserved Gln residue in the Q motif and a conserved Lys residue in the I motif are required for ATP binding and/or hydrolysis in some SF2 helicases (13, 10, 14), suggesting a similar role in chromatin remodelers.
As has been shown by in vivo experiments, the K798A mutation of the I motif eliminated the RNAstimulated ATPase activity of the yeast SWI2–SNF2 complex, whereby the swi2K798A allele exhibited a null phenotype in vivo (11, 12, 15). The mutations K798A in the yeast Swi2(p)/Snf2(p) ATPase and K798R in human BRG1 (Snf2 subfamily) (9) led to a reduced chromosomal HO-lacZ fusion gene and EF-1α promoter directed reporter gene expression, respectively (12, 16). Additionally, the corresponding mutation K999R in human CHD7 (Mi-2 subfamily) (9) resulted in a chromatin-remodeling deficiency of the enzyme (17). However, because of a lack of mechanistic details, these studies do not provide an explanation for the observed enzymatic defects. Moreover, the functional role of the Q motif in chromatin-remodeling enzymes is unknown so far.

We therefore examined the mechanistic roles of the Q and I motifs in ATP and nucleosome binding, in ATP hydrolysis and nucleosome remodeling in vitro. We further performed a comparative in vitro and in vivo study of these two motifs, using the well-characterized remodeling enzyme hBRG1 as a model system (18, 19). We replaced the highly conserved Gln758 residue (Q motif) or the highly conserved Lys785 residue (I motif) with similar ones or alanine. Surprisingly, the mutations did not alter the ability of the enzyme to bind ATP and also the basal ATPase activity was similar to wtBRG1. Furthermore, all mutants were still capable to interact with nucleosomes. However, their nucleosome-stimulated ATPase activity was lost. Accordingly, the chromatin-remodeling activity of all mutants was impaired. Interestingly, the cancer-relevant mutation of BRG1 L754F (Q motif) showed similar defects like the Gln758-mutant(s), arguing for a comparable loss of function. Because we can exclude a mutual interference of ATP and nucleosome binding, we postulate that both motifs stimulate the ATPase and chromatin-remodeling activity upon binding of BRG1 to nucleosomes, probably via allosteric mechanisms. Furthermore, our data argue that mutations of both motifs similarly affect the enzymatic functionality of BRG1, in vitro and in living cells. In line with the loss of function phenotype in vitro, exogenously expressed wtBRG1, but not BRG1 Q758A and BRG1 K785R, exhibited a tumor suppressor–like function (18, 20) in BRG1-deficient H1299 cells.

**Results**

**BRG1 Q and I motif mutants do still bind ATP**

To elucidate the functional roles of the Q and I motifs, a homology model of the BRG1 ATPase domain was determined (Fig. 1, B and C). The 3D model indicates that both motifs are in close proximity to the nucleotide: The side chain of Gln758 is oriented toward the N6 of the adenine base, and Lys785 is oriented toward the phosphate groups of ATP (Fig. 1, B and C). The distance of Gln758 and the N6 of the adenine base is 2.8 and 2.3 Å, respectively (Fig. 1C), suggesting a role for the Q motif in ATP binding and/or hydrolysis. Assuming a certain flexibility of the Lys785 residue and an interaction with the phosphate groups of ATPyS via complexed Mg2+ (13), a role in nucleotide binding and/or hydrolysis is also plausible for the I motif (Fig. 1, B and C). To test our hypothesis experimentally, we replaced Gln758 with Asn, Glu, Asp, or Ala (BRG1 Q758N, etc.) and Lys785 with Arg or Ala (BRG1 K785R, etc.). Interestingly, the K785R mutation is listed in the (COSMIC) database of somatic mutations in cancer (21). Because a further Q motif mutation, namely L754F, was present in this database, it was included into our study. All proteins were expressed and purified with high yields and purity from SF21 cells (Fig. 1D and data not shown). As shown before, the recombinant wtBRG1 protein (22) and also the mutant proteins form homomeric complexes on native polyacrylamide gels (Fig. S1), suggesting an overall structural integrity of the mutant proteins.

We next compared ATP binding, by measuring the binding of [γ-32P]ATP to the remodeling enzyme (Fig. 2A). The radioactive ATP filter-binding assays were performed as described (23) and revealed that wtBRG1 and all the mutants were still binding ATP in the absence of nucleosomes (Fig. 2A). Even though the filter-binding assays with remodeling enzymes display a high variability between biological replicates (data not shown), the presented binding signals nevertheless represent specific signals, because they were all significantly above the IgG background (Fig. 2A). Furthermore, the radioactive ATP signals decreased by competition with nonradioactive ATP (and less efficient with nonradioactive GTP) (Fig. 2A and Fig. S2). This suggests that the Q and I motifs may contribute to, but are not essential for, ATP binding.

**BRG1 Q and I motifs are required for nucleosome-stimulated ATPase activity**

Because the proteins were still capable of binding ATP (Fig. 2A), we next characterized them in a radioactive [γ-32P]ATP hydrolysis assay (Fig. 2B). The assay revealed that wtBRG1 and all the mutants exhibited comparable, basal ATP hydrolysis rates in the absence of nucleosomes, which were clearly distinguishable from chemical background ATP hydrolysis (Fig. 2B). These results rather suggest that all proteins bound ATP to a comparable level, which proposes again that the point mutations do not affect the binding and the basal mechanism/activity of ATP hydrolysis (Fig. 2). In contrast, upon addition of nucleosomes only wtBRG1 showed a significant increase in ATP hydrolysis (Fig. 2B). The ATPase activity of the mutant proteins was not stimulated by nucleosomes; it remained the same in the presence or absence of nucleosomes (Fig. 2B). Our data therefore propose a role for the Q and I motifs in the enhancement of the ATP hydrolysis rates of BRG1 in the presence of nucleosomes.

**The nucleosome association of BRG1 is independent of an intact Q or I motif**

The results shown in Fig. 2B leave open the question, whether the low ATP hydrolysis rate in presence of the nucleosomal stimulus might derive from the inability of the BRG1 mutants to bind nucleosomes. We therefore examined the
nucleosome binding of wtBRG1 and mutants by using biotinylated 77-NPS-77 nucleosomes (Fig. 3 and Fig. S3, A and B). Control experiments, including a preincubation step of BRG1 with “nucleosome free (circular)” plasmid DNA (see also “Experimental procedures”), revealed that the IP experiments reflect a specific binding of BRG1 to nucleosomes (Fig. S3 B). Using equimolar amounts of protein, we showed that all tested BRG1 proteins exhibited a similar binding to mononucleosomes, immobilized on streptavidin beads (Fig. 3), suggesting that the BRG1–nucleosome interaction is not affected by mutations in the Q or I motif. These data are supported by another set of IP experiments (Fig. S3C). The presence of ADP or the nonhydrolyzable nucleotide analogue ATPγS, both of which are competitive inhibitors of the ATPase domain and are therefore mimicking a “hydrolysis deficient state” (Fig. S3D), did not affect the association of the wtBRG1 enzyme with nucleosomes (Fig. S3C). Taken together, our data therefore show (i) that the

Figure 1. Structure and gel analysis of hBRG1. A, sequence of the ATPase, i.e. residues 726–1249 from BRG1 isoform 2 (Uniprot-ID P51532-2). The ATP-binding domain (light blue) and the helicase C-terminal domain (dark blue) are highlighted, and conserved blocks (cons. bl) and motifs (9) are indicated. The highly conserved Gln758 and Lys785 residues are printed in bold. B, homology model of the ATP-binding domain. The localization of ATPase motifs is indicated according to the color scheme shown on the right. ATPγS (black sticks) is docked into the ATP-binding site. C, a magnified view on the ATP-binding site, showing the orientation of the Q (violet) and I (red) motifs toward ATPγS. Gln758, Lys785, and ATPγS are shown as sticks, representing nitrogen (blue), sulfur (yellow), phosphorus (orange), oxygen (red), hydrogen (white), and carbon (green) atoms. Green dashed lines indicate hydrogen bonds between Gln758 and N6/7 of the nucleotide, and light blue lines indicate putative contacts between Lys785 and the phosphates of the nucleotide, which are presumably mediated by a Mg2+ atom (13, 10). The length of the respective bonds, including the hydrogen and the putative hydrogen acceptor, is listed. D, SDS-PAGE of BRG1 proteins. 1 μg of BRG1 proteins (wtBRG1, lanes 4 and 6; and mutants, lanes 1–3 and 7–10) were loaded on a 6% SDS-PAGE gel and stained with Coomassie (protein size marker, M, lane 5).
binding of BRG1 to nucleosomes is independent of the ATPase status and (ii) that the decreased ATP hydrolysis rates of BRG1 mutants in the presence of nucleosomes cannot be explained by their disability to bind nucleosomes (Fig. 3 and Fig. S3C).

**BRG1 Q and I motif mutants are impaired in chromatin-remodeling activity**

Because the mutants were still capable of binding and hydrolyzing ATP and of binding nucleosomes (Figs. 2 and 3), we next tested whether they were still able to move nucleosomes on dsDNA (Fig. 4A). We detected only for the WT protein a clearly visible remodeling activity on centrally (77-NPS-77) and end-positioned nucleosomes (0-NPS-77) (Fig. 4A). In the presence of ATP, wtBRG1 moved the 77-NPS-77 nucleosome from the center to the edge of the DNA strand and the 0-NPS-77 nucleosome from the border of the DNA fragment to the center (Fig. 4A). However, all BRG1 mutants failed to reposition the central nucleosome on 77-NPS-77 nucleosomes, but we observed an apparent remodeling activity of all mutant proteins on 0-NPS-77 nucleosomes (Fig. 4A). Even though this remodeling activity is weak as compared with wtBRG1, it is clearly distinguishable from the “background control” (Fig. 4A). These findings suggest that (in the case of the edge positioned nucleosomes) the basal ATP hydrolysis rates of the BRG1 mutants (Fig. 2B) were sufficient to move histone octamers on dsDNA (Fig. 4A). However, an efficient nucleosome remodeling only took place in the reactions containing wtBRG1, the sole protein being significantly stimulated in its ATP hydrolysis rate by nucleosomes (Fig. 2B). This suggests that either the current speed and/or the final amount of the released energy in the course of high ATP hydrolysis rates are required for “efficient” nucleosome translocation (Fig. 4C). Beyond that, our data propose that the Q and I motifs both are responsible for nucleosome-stimulated ATPase activity and linked to it chromatin-remodeling activity.

The interaction between BRG1 and nucleosomes does not enhance ATP binding

With respect to the nucleosome-binding experiments, we can exclude an influence of the ATPase domain status on the
BRG1–nucleosome interaction. We therefore raised the question of whether the association of wtBRG1 with nucleosomes might stimulate its ATP binding, resulting in turn in an increased ATP hydrolysis rate. To test this hypothesis, we performed ATP filter-binding assays with radioactively labeled ATP/H$_{9262}$/S (see above) in the presence and absence of nucleosomes (Fig. 4B). The detected radioactive signal in the presence of nucleosomes should increase significantly, if this hypothesis was correct. Using nucleosome to remodeler ratios reflecting those of the ATP hydrolysis assay (Fig. 2B), wtBRG1 did not show an enhanced ATP$_{9262}$/S-binding signal at rising concentrations of nucleosomes (Fig. 4B). These data argue against a nucleosome-dependent increase in the nucleotide-binding affinity of BRG1, suggesting in turn that the nucleosomal stimulation of the wtBRG1 ATP hydrolysis rate cannot be explained by an increase in its ATP-binding affinity. In combination with our results, showing ATP binding for all tested BRG1 proteins already in the absence of nucleosomes (Fig. 2), we also propose that the low ATP hydrolysis rates of the nucleosome-associated BRG1 mutants are not derived from a decreased ATP binding (Fig. 2B).

Altogether, our in vitro experiments finally show the independent binding of ATP or nucleosomes to BRG1, not influencing the association of the respective other substrate. Because the integrity of the Q and I motif are dispensable for the binding of ATP and nucleosomes, we can therefore specify the roles of the Q and I motifs in exclusively transmitting the stimulatory impulse of the (remodeler) bound nucleosome to the ATP hydrolysis center of the enzyme, stimulating in turn the chromatin-remodeling rate of the protein (Fig. 4C).

**WT BRG1 but not the mutant proteins exhibit tumor suppressor–like activities in H1299 cells**

We next asked, whether the chromatin remodeling–deficient mutants (Fig. 4A) would exhibit a phenotype in vivo.
The role of the Q motif in BRG1 remodeling

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A

B

C

mutants (& wt)

weakly
remodeled
nucleosomes

non-stimulated,
basal activity

edge-positioned
nucleosome

stimulated
activity

efficiently
remodeled
nucleosomes

BRG1

ATP

BRG1

ATP

BRG1

ADP

BRG1

ATP

BRG1

ADP
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and whether this would correlate with the loss of function phenotype in vitro. Previous work from other labs showed that reintroducing BRG1 (by stable or transient transfection) into various BRG1 mutant (tumor) cell lines, like Alab or SW-13, reversed their transformed phenotype by acting as a tumor suppressor (18, 20, 24). However, BRG1 with a point mutation in the “key lysine residue” of the I motif (KR) (16) did not exhibit these cell cycle regulatory functions in the mentioned cell lines (18, 20, 24). We therefore decided to compare the functional roles of the Q and I motifs by performing cell cycle analyses via flow cytometry for BRG1 K785R, BRG1 Q758A, and BRG1 L754F-expressing cells. We chose the BRG1- and p53-deficient H1299 cell line (Fig. 5A and Fig. 5B), which is derived from lung tumor tissue (18, 25). We used a transient expression system by transfecting constructs encoding C-terminal FLAG-tagged BRG1 proteins and a triple mCherry fusion protein, translated from an independent, internal ribosomal entry site (Fig. 5, A and B). All BRG1 proteins were expressed at comparable levels at the expected molecular mass level (Fig. 5A), and mCherry-positive cells exhibited a specific, punctuated nuclear immunocytochemistry signal for BRG1, rather excluding the nucleoli (Fig. 5B). This staining pattern is in accordance with data from the human Protein Atlas (www.proteinatlas.org) (26).  

Flow cytometric analyses were conducted with living H1299 cells 6 days post-transfection. mCherry-positive cells were gated, and their cell cycle profile was analyzed using Hoechst fluorescence staining. Expression of wtBRG1 resulted in a significant higher G1/G2 ratio compared with cells transfected with the mCherry control vector, suggesting that reintroducing wtBRG1 into H1299 cells consequently induced a more prominent enrichment of G1, resident cells than in the mock control (Fig. 5C). In contrast, the expression of BRG1 L754F, Q758A, or the K785R mutant, respectively, resulted in G1/G2 ratios that were not significantly different from transfactions with the control vector (Fig. 5C). However, the G1/G2 ratios of H1299 cells expressing the three BRG1 mutants differed significantly from the G1/G2 ratios of cells transfected with wtBRG1. Altogether, our flow cytometric analyses in H1299 cells support the findings of wtBRG1 acting in the presence of a tumor suppressor, keeping cells longer in the G1 phase of the cell cycle, as postulated in Refs. 20, 24, and 18 for the BRG1 mutant cell lines like Alab or SW-13. ATP hydrolysis and nucleosome remodeling deficient BRG1 proteins like BRG1 L754F, Q758A, and K785R fail to execute this tumor suppressor role, suggesting that both the Q and I motifs play comparable roles not only in vitro, but also in living cells. Interestingly, the Leu754 and Gln758 residues seem to have similar impact for the functionality of the Q motif.

Discussion

We elucidated the functional impact of the Q and I motifs for the human chromatin-remodeling enzyme BRG1. Against all odds, our data show that both motifs, being located in close proximity to the nucleotide, are not essential for ATP binding. We propose that the Q and I motifs transmit the BRG1-nucleosome association to the ATPase domain of the enzyme to stimulate its ATPase and chromatin-remodeling activity. Our functional and comparative analysis suggests for both motifs an equivalent relevance for the functionality of the enzyme.

The nucleosome and ATP binding of BRG1 are not affected by mutations in the Q and I motifs

The Q motif was proposed to mediate the protein dimerization process of the human FANCJ helicase (SF2B DEAH box helicase) (27). However, our native gel analyses (Fig. S1), revealing that the homomerization status of wtBRG1 and mutants is comparable, do not support this conclusion. The homotypic multimerization of BRG1, we observed in our experiments, correlates with gel filtration data from Ref. 22 showing that recombinant wtBRG1 seems to form homomers in a molecular mass range between 500 kDa and 2 MDa. We therefore suggest that the Q and I motifs of BRG1 are not involved in mediating homomeric interactions of this enzyme. A similar conclusion was proposed by Fitzgerald et al. (28) for yeast Iswi2 (Iswi subfamily) (9). The authors show that the presence of ATPγS and ADP does not interfere with the heterodimeric character of the enzyme (28). Interestingly, the human DEAH box helicase ChlR1 (SF2 iron–sulfur) (29) works predominantly as a monomer and this “single species status” is also unaffected by mutations in the Q motif (30).

Figure 4. Chromatin-remodeling activity of wtBRG1 and mutants and nucleotide binding to wtBRG1 in the presence and absence of nucleosomes-working model for the function of the Q and I motifs. A 130 nM edge positioned mononucleosomes [upper panel, 0-NPS-77; lower panel, 77-NPS-77] were incubated with 250 or 500 nm enzyme with/without 1 nm ATP (left and right upper panel, lanes 2–4 and 14–16 with wtBRG1 and lanes 5–12 and 17–24 with indicated BRG1 mutants; lanes 1 and 13 with input nucleosome fraction and left and right lower panel, lanes 26–28 and 38–40 with wtBRG1 and lanes 29–36 and 41–48 with indicated BRG1 mutants; lanes 25 and 37 with input nucleosome fraction). Changes in nucleosome position were resolved on native 0.4 TBE, 6% PAA gels. The intensity of bands in each lane was quantified using Fuji Film multigauge software. Signal intensity profiles of selected gel lanes are highlighted (41–48). This suggests that both the current speed and/or the final amount of the released energy in the course of high ATP hydrolysis rates are required for “efficient” nucleosome translocation (initial, edge-positioned nucleosomes are presented in dark gray; weakly (left) and efficient (right) remodeled nucleosomes are displayed in distinct, lighter shades of gray). Because we can exclude an influence of ATP or nucleosomes on the binding of the respective other substrate to BRG1, we therefore specify the function of both motifs as follows: the Q and I motifs are transmitting the stimulatory impulse of the remodeler associated nucleosome to the ATP hydrolysis center of the enzyme, stimulating in turn the chromatin-remodeling rate of the protein.

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Figure 5. Western blotting, immunocytochemical, and cell cycle analysis of H1299 cells expressing wtBRG1 and mutant proteins. A, 48 h after transient transfection with the indicated plasmids, H1299 cells were loaded on 6.5% SDS gels, which were subsequently transferred to PVDF membranes via semidy blotting. The PVDF membrane was cut into three pieces, which were incubated with the indicated antibodies. Tubulin served as a loading control. B, 48 h after transient transfection with the indicated plasmids, H1299 cells were fixed in 4% PFA, Triton X-100-permeabilized, and immunostained with an “anti-FLAG” primary antibody in combination with an Alexa 488-labeled secondary antibody. The nuclear DNA was colored via DAPI. The cells were finally mounted in PBS/glycerol, and pictures for the indicated fluorophores were taken at the fluorescence microscope Axiovert 200M. Scale bar, 20 μm. C, 24 h after transient transfection with the indicated plasmids, H1299 cells were seeded (subconfluent) for a period of 6 days on P150 plates. Six days after transfection, the cells were labeled with Hoechst, trypsinized, filtered, and finally analyzed with a CyFLOW space flow cytometer (Partec). The ratio between the cell numbers between the G1 and G2 populations (see “Experimental procedures”) from five or six biological replicates was calculated (G1/G2 ratio) and plotted as box-plot diagrams, using the software Origin 2017 (vc, vector control = empty mCherry vector). The median is shown as a line, and the average is shown as a green box and is displayed right to the box. The statistical significance was calculated in Origin 2017 software, performing a t test with two samples, mean 1 – mean 2 < 0, significance-niveau 0.05, assuming heteroscedastic variance (Welch Korrektur) with *, p < 0.05; **, p < 0.01; and ***, p < 0.001 and not significant (n.s.).
The role of the Q motif in BRG1 remodeling

Beyond that, we were also studying the influence of the ATPase status on the chromatin-binding behavior of hBRG1. From the nucleosome-binding data we conclude that neither the Q motif nor the I motif were required for the nucleosome binding process (Fig. 3). For chromatin-remodeling enzymes it is still controversially discussed whether the association of the enzymes with DNA or nucleosomes requires a functional ATPase domain. Although FRAP experiments revealed slower recovery kinetics of the YFP-tagged BRG1 KR mutant (corresponds to our BRG1 K785R mutant) compared with the WT protein on the MMTV-LTR array in 1361.5 cells (31), arguing that an intact ATPase domain is required for chromatin association, other laboratories obtained opposing results. Xie et al. (32) observed comparable chromatin affinities of CHD4 K757R mutant (I motif) and wtCHD4 (Mi-2 subfamily) (9) to the rDNA locus in ChIP experiments. Cross-linking experiments showed that ADP even slightly improved the binding of yeast INO80 (Ino80 subfamily) (9) to (70N0) nucleosomes (33). Even though we cannot rule out that our IP experiments do not allow the detection of minor changes in binding affinity, our data are in good agreement with the results from Refs. 32 and 33. We propose an independence between nucleosome association and ATPase domain status. Furthermore, slight differences in nucleosome-binding affinity would not explain the drastic effects on ATPase stimulation and chromatin remodeling (Figs. 2B and 4A).

Crystal structures indicate that the Q and I motifs are in close proximity to the bound nucleotide (Fig. 1, B and C), which suggests a role in nucleotide binding for both motifs (13, 10, 14). However, we showed that the two motifs are not essential for nucleotide binding (Fig. 2). This is also true for other SF2 helicases, like hChR1, hFANCJ, and bovine ADAAD (SMARCAL1 subfamily) (9, 27, 30, 34). However, there are also cases in which both motifs are involved in ATP binding. A yeast Ded1 helicase Q motif mutant showed an increased RNA dependent $K_m$ value for ATP, and a S. cerevisiae translation initiation factor elf4A I motif mutant (all DEAD box SF2 helicases), for example, showed a loss of ATP binding (13, 35).

The Q and I motifs of human BRG1 transmit the stimulatory effect of nucleosome binding to the ATPase domain

Because our data argue that both motifs are not essential for nucleosome and nucleotide binding (Figs. 2 and 3), we tested whether the association with one substrate might change the binding affinity of the respective other substrate. There are reports about an accelerated binding of ATP to the Cdc6 protein (ATPase) upon its association with ORC (origin recognition complex), an increased ATP affinity of ADAAD in the presence of sDNA (stem-loop DNA), or slightly lower $K_m$ values for ATP in the presence of M13ssDNA for FANCJ (27, 34, 36). On the other hand, it is known that enzymes like ADAAD exhibit lower $K_m$ values for their sDNA substrate in the presence of ATP than in its absence (34). However, neither ATP (more precisely $ATP_\gamma S$) nor nucleosomes stimulated the binding of the respective other substrate to wtBRG1 in our hands (Fig. 4B and Fig. S3C).

Because the basal (nonstimulated) ATPase rates remained unaffected upon substitution of the conserved Gln and Lys residues of both motifs (Fig. 2B), we therefore propose the following role for the two BRG1 motifs: Transmitting the stimulatory effect of the remodeler associated nucleosome on to the ATPase activity and linked to it chromatin-remodeling activity. One can easily imagine that conformational changes of the enzyme are involved in this “transmitter process.” It was for example shown for Chaetomium thermophilum INO80 (Ino80 subfamily) (9) that the subunits Arp5–1es6 couple the ATPase activity to nucleosome sliding by contacting at the same time DNA and histone octamer, thereby adopting at least two conformational states (37).

Interestingly, two laboratories came to similar conclusions about the function of these two motifs in the two SF2 helicases FANCJ (27) and ADAAD (34, 38), exhibiting unlike BRG1 higher ATP affinities in the presence of the corresponding DNA substrate (see above) (34, 27). Nevertheless, in ADAAD both motifs are exclusively responsible for (DNA-stimulated) ATP catalysis, but not for ATP binding, whereby the Q motif seems to play a particular, regulatory role for the catalytic efficiency (34, 38). Regarding FANCJ, the authors observed a more or less similar nonstimulated (basal) ATPase activity of the WT, Q-mutant, and I-mutant proteins (determined via $k_{cat}$). Upon M13DNA addition, the ATPase rate (determined via $k_{cat}$) of the FANCJ WT protein was stimulated much more intense compared with the one of the Q mutant. However, the ATP hydrolysis rate of the I mutant remained unchanged, suggesting an imbalance in the functional relevance of the Q and I motifs for the stimulation of the ATP hydrolysis of FANCJ via M13DNA. Similarly, Tanner et al. (13) presented on the one hand a S. cerevisiae elf4A I mutant (K71A), which did not bind ATP, and on the other hand a Q motif mutant (Q48E), which clearly bound ATP. In contrast to the results shown for FANCJ and yeast elf4A, we propose by our in vitro and in vivo experiments that both motifs exhibit equivalent roles for the enzymatic functionality of BRG1.

BRG1 L754F (Q motif)—a putative cancer driver mutation?

Our in vitro and in vivo studies showed that the naturally occurring, cancer-relevant BRG1 L754F mutant (21) exhibited the same enzymatic defects like BRG1 mutants with mutations on the conserved Gln$^{758}$ and Lys$^{785}$ residues (Figs. 2B, 4A, and 5C). Interestingly, this leucine is highly conserved in the Snf2 family of SF2 helicases (Fig. S5) (9). However, in DEAD box helicases (SF2 family), this position is occupied by a proline residue, forming hydrophobic interactions with a well conserved phenylalanine, which is commonly located 17 residues N-terminal of the Q motif (Fig S5) (13). Nevertheless, the proline-to-leucine mutation in S. cerevisiae elf4A only caused marginal growth defects of the transformed, double-deleted yeast strain (13). An alignment of the protein sequences of three human chromatin-remodeling enzymes from three different Snf2 subfamilies, CHD3 (Mi-2), Snf2h (Iswi), and BRG1 (Snf2) (9) did not reveal a phenylalanine in an equivalent distance to the Q motif (Fig. S5). Interestingly, Prp5 (DEAD box helicase), which also has a leucine residue four positions N-terminal of the highly conserved Gln residue, also lacks a phenylalanine (13). One could therefore speculate about mutual dependences of the considered residues. Our findings and previous ones (13)
suggest different effects for the functionality of the Q motif caused by the proline and leucine residues under study. Most importantly, the naturally occurring L754F mutation in BRG1 results in a loss of function of recombinant BRG1 L754F (Figs. 2 and 4A). In accordance with this, BRG1-deficient H1299 cells, expressing BRG1 L754F or BRG1 Q758A and BRG1 K785R, exhibited significantly lower $G_1/G_2$ ratios than H1299 cells, expressing wtBRG1 (Fig. 5C). These data propose that the two cancer-relevant BRG1 mutations L754F and K785R (21) reflect the impact of cancer driver mutations (39).

**In contrast to wtBRG1, BRG1 L754F, Q758A, and K785R do not exhibit tumor-suppressor–like function in H1299 cells**

According to our cell cycle analyses with BRG1-deficient H1299 cells, the functional integrity of the ATPase domain of reintroduced BRG1 is necessary for the observed accumulation of cells in the $G_1$ phase. The mutants with nonfunctional ATPase domains do not change the cell cycle distribution, demonstrating the importance of this domain for the *in vivo* effect of BRG1 (Fig. 5C). Many (human) cell types commit to a complete cell cycle at the $G_1$–$S$ transition, because early and late $G_1$ phase exhibit $G_1$ control points, one of which is the so-called “restriction point,” regulating cell cycle progression (40). Therefore, an accumulation of cells in $G_1$ is in accordance with a tumor suppressor function for BRG1 in H1299 cells (40). This function is apparently abolished in ATPase-deficient mutants (Fig. 5C). Interestingly, Romero *et al.* (41) showed that wtBRG1 expressing H1299 cells form significantly less colonies than control cells or cells expressing a BRG1 protein with a lack of function mutation (W764R, in the ATPase domain). According to Knudsen and Angus (42), these kinds of results also reflect a tumor suppressor activity of BRG1. Taken together, our experiments and those of Romero *et al.* (41) are also in good agreement with data from several other laboratories (18, 20, 24, 43), arguing as well for a role of BRG1 as a tumor suppressor. Similar to our studies, overexpression of wtBRG1 in BRG1-deficient SW-13 cells (adrenal carcinoma cell line) and Alab cells (derived from breast tumor tissue) induced an increase of cells in the $G_1$ phase and a decrease of cells in the $S$ and $G_2$ phases (16, 18, 43). In Alab cells, this effect was not observed in mock controls or when cells were transfected with the BRG1 KR mutant (corresponds to our K785R mutant) (16, 18). Beyond that, 100% of Alab cells, stably expressing wtBRG1, showed a significantly flattened cell shape compared with control cells (18). In combination with the cell cycle analysis data, these results deliver a strong argument for the antimitogenic/tumor suppressor activity of wtBRG1 in Alab cells (42). This tumor suppressor activity in Alab cells may be partly mediated by the down-regulation of E2Ftargetgenesandheup-regulationofmRNAsfortheclincal-dependent kinase inhibitors p15 and p21 (44). The latter protein was also shown to be up-regulated in BRG1 expressing SW-13 cells (43), p15 and p21 are cyclin-dependent kinase inhibitors for Cdk 4 and 6, and both can act as negative regulators of the $G_1$–$S$ transition (45, 46).

However, the effect of BRG1 expression does not cause a complete stop of cell cycle progression (Fig. 5C). This can be also seen by electric cell–substrate impedance sensing (ECIS) measurements (Fig. S6 and see also “Experimental procedures”). Because it has been shown that the decrease in total capacitance is linearly correlated with the increase in electrode surface coverage with cells (47), cell proliferation can be monitored by analyzing the normalized capacitance $C_{\text{norm}}$ (instead of the complex impedance value) at a sensitive AC frequency of 32 kHz (47) (Fig. S6A). The slope values of the resulting proliferation curves indicate that H1299 cells expressing wtBRG1 are proliferating at a similar rate as cells expressing only mCherry (Fig. S6, A and C). However, BRG1-overexpressing cells spent more time in $G_1$, as revealed by the flow cytometric analyses (Fig. 5C). This supports the idea of a BRG1-induced growth delay by inhibiting or preventing the $G_1$–$S$ transition. Under cell culture conditions, the cell doubling time and thereby overall growth of cell mass might be similar to BRG1-deficient cells because compensatory mechanisms will lead to the shortening of other cell cycle phases as observed in other cell types (48). *In vivo*, in the context of a multicellular environment, any constriction of the progression into the $S$ phase should be a tumor suppression function. A loss of this tumor suppressor function could contribute to the onset of uncontrolled proliferation, thereby promoting cancer formation.

**Outlook**

It will be challenging to elucidate in detail the allosteric effects, which are involved in transferring the BRG1–nucleosome association on to the stimulation of ATPase and chromatin-remodeling activity. However, because many chromatin-remodeling enzymes are related to cancer and other diseases, a better understanding of the chromatin remodeling mechanisms would also facilitate the development of novel therapeutic strategies.

**Experimental procedures**

**Materials and methods**

The following reagents were used: mCherry antibody (orb66657 biorybt); rat anti-DYKDDDDK antibody (200474, Agilent Technologies); anti-BRG1 antibody (ab1110641, Abcam); anti-α-tubulin (rabbit) antibody (600-401-880S, Rockland); anti-H2B (07-371, Upstate-Millipore); mouse anti-p53 antibody (sc-126, Santa Cruz (DO-1)); peroxidase-conjugated AffiniPure goat anti-rabbit IgG (H+L) (111-035-144, Jackson ImmunoResearch); peroxidase-conjugated AffiniPure goat anti-mouse IgG (H+L) (111-035-146, Jackson ImmunoResearch); Alexa Fluor® 488 goat anti-rat IgG (H+L) (A-11006, Invitrogen or Thermo Fisher Scientific); streptavidin, immobilized on agarose CL-4B (Fluka/Sigma–Aldrich); ATP ultrapure (Jena Bioscience); ADP ultrapure (Cell Technologies); ADPγS (Jena Bioscience); [$\gamma\text{-}^{35}\text{S}]ATP (PerkinElmer); [$\gamma\text{-}^{32}\text{P}]\text{ATP (Hartmann Analytic); NuPAGE }4\text{–}12\% \text{ Bis-Tris gels (Thermo Fisher Scientific); anti-FLAG M2-agarose beads (Sigma–Aldrich); DAPI (Sigma–Aldrich); Amicon Ultra-4 centrifugal filter units }500\text{ µl of 10,000 molecular mass cutoff (Merck Millipore); FuGENE® HD transfection kit (Promega); complete EDTA-free protease inhibitor mixture (Roche); benzonase (E1014-5KU, Sigma); glass coverslips (Ø = 12 mm) (Roth); 24-mm × 60-mm cover glasses (Roth); 76-mm × 26-mm glass microscope slides (Roth); PEI-cellulose F plates (Merck Millipore); Amersham Biosciences Protran premium

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The role of the Q motif in BRG1 remodeling

nitrocellulose blotting membrane 0.45-μm pore size (GE Healthcare); PVDF Immobilon-P transfer membrane 0.45-μm pore size (Merck Millipore); Super Signal West Dura Extended Duration Substrate (Thermo Fisher Scientific), NativePAGE 4–16% Bis-Tris protein gels (Thermo Fisher Scientific); Sf-900\textsuperscript{TM} II SFM (1×) medium (Thermo Fisher); Dulbecco’s modified Eagle’s medium, low glucose [1 g/L], GlutaMAX\textsuperscript{TM}, I, medium (Thermo Fisher); penicillin/streptomycin (10,000 units/ml) (Gibco–Thermo Fisher Scientific); 0.5% trypsin-EDTA (10×) (Gibco–Thermo Fisher Scientific); fetal calf serum (Gibco); TNF-α (Sigma–Aldrich); HEPES (Sigma–Aldrich); 1 mM HEPES (Gibco); fetal bovine serum (dialyzed) (Thermo Fisher); Partec CellTrics (Grenoble, Grenoble Cedex 9, France) and grown in LB medium. H1299 cells were grown in Dulbecco’s modified Eagle’s medium, low glucose (1 g/liter), GlutaMAX\textsuperscript{TM} I, medium (Thermo Fisher); penicillin/streptomycin (10,000 units/ml) (Gibco–Thermo Fisher Scientific); 0.5% trypsin-EDTA (10×) (Gibco–Thermo Fisher Scientific); fetal calf serum (Gibco); TNF-α (Sigma–Aldrich); HEPES (Sigma–Aldrich); 1 mM HEPES (Gibco); fetal bovine serum (dialyzed) (Thermo Fisher); Partec CellTrics\textsuperscript{®} filter (50 μm) (Partec); poly-9

Plasmids and constructs

The sequence for WT human BRG1 (with C-terminal FLAG epitope) was obtained in form of pFastBac BRG1 F (encoding transcription variant 6; NM_001128847) from Dr. Robert Kingston (Department of Molecular Biology, Simches Research Center, Boston, MA). The motif (Walker I) and Q motif point mutations were generated by mutagenesis PCR. The wtBRG1 and mutant cDNAs (all with C-terminal FLAG epitope) were furthermore subcloned in pSR-948-CMV-pIRES-3x Cherry (in house production) or in case of the BRG1 K785R mutant in pIRES2-eGFP (Clontech). The pPCRScript\textsubscript{slog} l-gla75, encoding the nucleosome positioning sequence, used for the assembly reaction (see below), was described in detail in Ref. 49. The pT11 plasmid DNA, used for stopping remodeling reactions (see below), was obtained from Dr. Joachim Griesenbeck (Biochemistry III, University of Regensburg, Regensburg, Germany). It was first described in Ref. 50. All plasmids were sequence-verified.

Bacterial strains and cell lines

DH10 BacYFP were obtained from Dr. Imre Berger (EMBL Grenoble, Grenoble Cedex 9, France) and grown in LB medium. SF21 cells were obtained from Thermo Fisher and grown in Sf-900\textsuperscript{TM} II SFM (1×) medium at 27 °C on horizontal shakers (100 rpm) in glass Erlenmeyer flasks. Human H1299 cells (ATCC\textsuperscript{®} CRL-5803\textsuperscript{TM}), a nonsmall cell lung cancer cell line (derived from metastatic site: lymph node), were a kind gift from Prof. Dr. Harald Wodrich (Laboratoire de Microbiologie Fondamentale et Pathogénicité, University of Bordeaux, Bordeaux, France). H1299 cells were grown in Dulbecco’s modified Eagle’s medium, low glucose (1 g/liter), GlutaMAX\textsuperscript{TM} I, medium, supplemented with 10% fetal calf serum at 37 °C with 5% CO\textsubscript{2}. SW-13 cells (ATCC\textsuperscript{®} CCL-105\textsuperscript{TM}), an adrenal carcinoma cell line, was a kind gift from Prof. Dr. Felix Beuschlein and Dr. Constanze Hantel (Medizinische Klinik und Poliklinik IV Endokrinologische Forschung, Ludwig-Maximilians-Universität, Munich, Germany). SW-13 cells were grown like H1299 cells.

Nucleosome assembly

The nucleosome assembly strategy and procedure are described in detail in Ref. 49. In brief, for the assembly of centrally (77-NPS-77) and end-positioned (0-NPS-77) mononucleosomes, with 77 or 0 bp comprising linker DNA, primer combinations from (49) were used to amplify the respective DNA sequences from a BglIII-digested and gel-purified DNA fragment of pPCRScript\textsubscript{slog}1-gla75. In case of the biotin-labeled, centrally (77-NPS-77) positioned nucleosome, the reverse primer (GTACAGAG AGGGAGAGTCACAAAAC) was labeled at its 5′-end with biotin. The PCR-amplified NPS sequence was ethanol/NH\textsubscript{4}-acetate precipitated and dissolved in water. The nucleosomes were generated by salt gradient dialysis, using chicken histone octamer:DNA ratios that varied from 0.6:1 to 1:1 (49). The mononucleosome concentration was determined by dividing the amount of DNA, used for the assembly reaction, through the total reaction volume after dialysis. Mononucleosomes for ATPase assays with BRG1 (see below) were dialyzed against low salt buffer with 80 mM salt after the assembly procedure.

Virus generation

Recombinant human wtBRG1 and its mutants were generated according to Refs. 49 and 51. In brief, pFastBac vectors (see above), encoding C-FLAG wtBRG1 or mutant proteins, were transformed in DH10Bac YFP cells. The bacmids, extracted from positive DH10BacYFP (white) clones, were subsequently transfected via FuGENE HD into SF21 cells, following the manufacturer’s instructions. 60–72 h after transfection, the V\textsubscript{0} generation virus was harvested, which in turn was used to produce the V\textsubscript{1} generation virus. The latter virus generation was used for large scale infections of 300 – 400-ml cultures.

Purification of FLAG-tagged BRG1 proteins from insect cells

The purification for C-terminal FLAG-tagged human wtBRG1 and its mutant proteins was adapted from the protocol in Ref. 49. In brief, human wtBRG1 and its Q and I motif mutants were purified from 1–2 × 10\textsuperscript{8} SF21 cells. The FLAG epitope-tagged protein expressing cells were lysed in 20 mM Tris, pH 7.6, 500 mM KCl, 1.5 mM MgCl\textsubscript{2}, 0.5 mM EGTA, 0.1% IGEPAL-CA630, 1× protease inhibitor mixture. After lysis, 0.6 μl of benzonase were added, and the cell lysates were subsequently incubated with anti-FLAG M2-agarose beads for 3 h on a rotating wheel at 4 °C. Afterward, the beads were transferred to gravity flow columns and subsequently treated with 100 column volumes wash buffer (20 mM Tris, pH 7.6, 500 mM KCl, 1.5 mM MgCl\textsubscript{2}, 0.5 mM EGTA, 0.1% IGEPAL-CA630) and subsequently equilibrated with wash buffer, containing 333 mM KCl. The elution was performed in 20 mM Tris, pH 7.6, 333 mM KCl, 1.5 mM MgCl\textsubscript{2}, 0.5 mM EGTA, 0.1% IGEPAL-CA630, 1× protease inhibitor mixture, 500 ng/μl FLAG peptide by performing in a total of five elution steps with one column volume per elution step. In case it was necessary, the protein fractions were subsequently pooled and concentrated via ultrafiltration columns to the desired concentration. At this step, the proteins were supplemented with glycerol (final concentration, 10%) and frozen in liquid nitrogen for final storage at −80 °C.
Nucleosome remodeling reaction

Nucleosome remodeling assays were performed in 20 mM Tris-HCl, pH 7.6, 80–120 mM KCl, 1.5 mM MgCl₂, 0.5 mM EGTA, 10% glycerol, 1 mM ATP (or 1 mM ATP/S) at 30 °C for 60 min in 10–11 μl of reaction volume. Each reaction contained mononucleosomes in end concentrations of 120–130 nM (77-NPS-77 or 0-NPS-77). The final concentration of recombinant nucleosome remodeling enzymes varied from 250 to 500 nM. Where indicated, nucleosome remodeling reactions were supplemented with 0.25, 0.5, and 1 mM ATP/S (in the presence of 1 mM ATP). The enzymatic reactions were stopped by adding 300–1000 ng of competitor (plasmid) DNA for 5 min at 30 °C. The nucleosome movements were visualized by supplementing the reactions with gelatin (4–5%) and loading them on 5% or 6% native 4× TBE PAA gels, which were subsequently stained with ethidium bromide.

Nucleosome immobilization

For immobilizing centrally positioned nucleosomes, 1.053 μg of biotinylated nucleosomes (see above) were incubated at 30 °C for 30 min in 20 mM Tris-HCl, pH 7.6, 150 mM KCl, 1.5 mM MgCl₂, 0.5 mM EGTA, 10% glycerol, 0.1% IGEPA-C630, 200 ng/μl BSA, 1% gelatin on a horizontal shaker (500 rpm) with 7.5 μl of pure streptavidin agarose CL-4B beads in a total volume of 50 μl. Afterward, the beads were washed three times in 100 μl of 20 mM Tris-HCl, pH 7.6, 150 mM KCl, 1.5 mM MgCl₂, 0.5 mM EGTA, 10% glycerol, 0.1% IGEPA-C630. After removing the supernatant quantitatively, the beads were supplemented with 527 nM wtBRG1 or BRG1 mutant proteins in 20 mM Tris-HCl pH 7.6, 150 mM KCl, 1.5 mM MgCl₂, 0.5 mM EGTA, 10% glycerol, 1× proteinase inhibitor mixture in a total volume of 50 μl and incubated for 1 h at 30 °C (500 rpm on a horizontal shaker). To test the influence of nucleotides, 1 mM of ADP or ATP/S were added to the remodeler containing IP reactions. In some of the experiments, the remodeling enzymes were preincubated for 30 min with 3.6 μg of nucleosome-free pT11 plasmid DNA (preincubation volume, 42.5 μl). Afterward, the beads were washed again three times in 100 μl of 20 mM Tris-HCl, pH 7.6, 150 mM KCl, 1.5 mM MgCl₂, 0.5 mM EGTA, 10% glycerol, 0.1% IGEPA-C630 and finally resuspended in 50 μl of 1× Laemmli buffer. 20% of the IP reactions were loaded on NuPAGE 4–12% Bis-Tris gels for further analysis. The input samples contained 0.21 μg of nucleosomes, 1% (final concentration) gelatin, 2 μg of BSA, or 0.96 μg of remodeler. The gels were analyzed via Coomassie stain, silver stain, or Western blotting.

Nucleotide filter-binding assays

To assay ATP binding, 200 nM of FLAG-tagged BRG1 WT and mutants were incubated with 0.375 μCi of [γ-32P]ATP in 2.8 mM MgCl₂, 20 mM HEPES, pH 7.6, 120 mM KCl, 0.4 mM EDTA, 10% glycerol in a volume of 15 μl at 30 °C for 30 min. As control reactions, [γ-32P]ATP was also incubated with buffer only and with equivalent amounts of rabbit IgG or BSA. A 45-mm-diameter piece of 0.45-μm nitrocellulose membrane was installed in a vacuum filtration system (Nalgene) and washed with 20 ml of buffer A (20 mM HEPES, pH 7.6, 100 mM KCl, 0.4 mM EDTA, 10% glycerol). 4.5 μl of the binding reactions were spotted on the nitrocellulose membrane under vacuum, and the membrane was washed with 150 ml of buffer A. The membrane was dried at 65 °C immediately afterward and placed on a phosphoimaging screen. After exposure overnight, screens were readout on a Typhoon FLA 9500 imager and quantified using Fuji Film Multi Gauge. An average and standard deviation were calculated from the triplicate spot signals. In rare cases where one signal deviated by more than 50% from the average of the other two in the triplicate, this outlier was disregarded, and the average was calculated from the remaining two spot signals. Calculations were done in Microsoft Excel using the “average” and “stdev” functions.

To test ATP binding in presence of nucleosomes, we added 600 nM symmetrically positioned mononucleosomes with 77 bp of free DNA on each side of the nucleosome core particle. To avoid hydrolyzing the [γ-32P]ATP, it was replaced with 0.375 μCi of [γ-35S]ATP.

ATPase assay

Recombinantly purified BRG1-FLAG and its respective mutants (250 nM) were incubated with 400 nM 77-NPS-77 mononucleosomes in 20 mM Tris- HCl pH 7.6, 120 mM KCl, 1.5 mM MgCl₂, 0.5 mM EGTA, 10% glycerol in the presence of 500 μM ATP and 0.1 μCi of [γ-32P]ATP for 40 min at 30 °C. Released 32P was separated from nonhydrolyzed [γ-32P]ATP by TLC on PEI-cellulose F plates (mobile phase: 50% acetic acid, 0.5 mM LiCl). TLC plates were dried immediately afterward at 65 °C and exposed on phosphoimaging plates. The plates were readout on a Typhoon FLA 9500 instrument, and spots were quantified using the Fuji Film multi gauge software.32P to [γ-35S]ATP ratios and averages were calculated in Microsoft Excel. Standard deviations originate from three technical replicates and were calculated using the “stdev” function. The experiment shown is representative for three independent experiments done with two independent protein preparations.

Native PAGE

Oligomerization of BRG1 WT and mutants was assayed by 4–16% NativePAGE gels from Thermo Fisher Scientific. 3 mm of BRG1 WT and mutants were incubated in 20 mM Tris-HCl, pH 7.6, 150 mM KCl, 1.5 mM MgCl₂, 0.5 mM EDTA, 1% glycerol, 1× proteinase inhibitor mixture and 0.01% IGEPA-C630. Afterward, the beads were washed three times in 100 μl of 20 mM Tris-HCl, pH 7.6, 150 mM KCl, 1.5 mM MgCl₂, 0.5 mM EGTA, 10% glycerol, 0.1% IGEPA-C630 and finally resuspended in 50 μl of 1× Laemmli buffer. The concentration of the IP reactions was adjusted to 30 μg of protein and loaded on a NuPAGE 4–12% Bis-Tris gel. The gel was run on a 12% gel using 2.4 μl of protein and electrophoresis was continued until the bromophenol blue marker reached the 12% gel. The gel was dried at 65 °C and exposed on phosphoimaging plates. The plates were readout on a Typhoon FLA 9500 instrument, and spots were quantified using the Fuji Film multi gauge software. The ratios of [γ-35S]ATP were calculated using Microsoft Excel.

Flow cytometric measurements and cell cycle analysis

For cell cycle studies 500,000 H1299 cells were seeded in T10 plates. 24 h later, the cells were transfected using FuGENE HD with CMV pIRES 3x Cherry constructs (see below under “Immunocytochemistry”), encoding for C-FLAG wtBRG1 or Q and I motif point mutants. One day after the transfection, the cells were trypsinized and seeded at a density of 500,000 cells per dish.
The role of the Q motif in BRG1 remodeling

of 200,000–500,000 cells/P150 dish in medium, supplemented with penicillin/streptomycin and incubated for 6 days (in case, where it became necessary, the cells were trypsinized in between the 6-day incubation time to keep them in a subconfluent state). Six days after transfection the (subconfluent) cells were incubated in fresh medium, containing 3 μg/ml Hoechst (33342) for 1 h. Afterward, the cells were trypsinized and finally resuspended in FACS buffer (1× PBS (Ca²⁺ and Mg²⁺ free), 25 mM HEPES, 2 mM EDTA, 2% dialyzed fetal calf serum, 50 μg/ml DNase I, 3 μg/ml Hoechst 33342) at a density of 5–10 million cells/ml. Afterward, the cells were kept on ice and filtered through a CellTrics® filter into polyurethane round bottom tubes shortly before the measurements. Flow cytometric measurements were performed at room temperature with the CyFlow space flow cytometer (Partec). Hoechst was excited with a 365-nm UV laser—LED and detected via the FL2-bandpass filter BP 455/50 (FL2 channel), and mCherry was excited with a 561-nm yellow laser and detected with a FL3-bandpass filter BP630/75. To gate cells, the forward scatter (FSC) and side scatter (SSC) were recorded by using the 488-nm blue solid state laser for excitation and a longpass filter IBB 488 for detection.

The data recorded by the CyFlow space flow cytometer (Partec) were loaded into the FCS-Express program (De Novo Software). Two gates were created by using 2D plots to select single cell populations. In the first plot, forward scatter (FSC) and side scatter (SSC) were displayed and the region in which the signal combination from intact cells accumulate was set as the “cells” gate. In the second plot, the intensity of the Hoechst signal was plotted against the peak width of the Hoechst signal. A gate (“DNA”) was selected that contains the signal from single cells and discarding very low Hoechst intensities resulting from cell fragments or other smaller particles. The combination of both gates resulted in the “cells-DNA” gate.

Data analysis was performed with the software Origin 2017. In a first step, the data containing the information of the FL2 channel (Hoechst staining) and FL3 channel (Cherry signal) of the “cells-DNA” gate was imported into Origin 2017. The background signal of the Cherry fluorescence was determined by the analysis of non-transfected cells and the FL3 values in which 99.5% of these cells reside was set as Cherry-negative. The start of Cherry-positive cells was set by taking the maximal background level and adding 5% to this number. The cells were then sorted into positive and negative population based on these numbers.

A cell cycle profile (Hoechst histogram plot) of the “negative” cells was then calculated and plotted by analyzing the frequency of cells containing DNA content with a step (bin) size of 50. A typical profile contains two peaks. The first peak with a lower DNA content contains cells that are mostly in the G₁ phase, and there is a second peak where most cells reside in the G₂ phase. The maxima of the two peaks are then calculated. Based on this peak analysis, the cell population was split into G₁ and G₂ cells. G₁ was set as the area of the Hoechst fluorescent values that start 200 points before the G₂ peak and ends at the G₁ peak. For G₂, the area was set to start at the G₂ peak, and it ends 300 Hoechst values after the peak. By this method, any overlap of S-phase cells is minimal. Based on these gates, the population of Cherry-positive and Cherry-negative cells was again split into G₁ and G₂ populations. Finally, the ratio between the cell numbers between the G₁ and G₂ population from five or six biological replicates was calculated (G₁/G₂ ratio) and plotted as box-plot diagrams, using the software Origin 2017. The statistical significance was calculated in Origin 2017 software, performing a “t test” with two samples, mean 1 – mean 2 o.05; and *** p < 0.001.

Immunocytochemistry

For analyzing the intracellular localization and expression of human BRG1 and its Q and I motif mutants, 187,500 cells were seeded in a P6 dish 1 day before the transfection. The cells were transfected according to the manufacturer’s protocol of the FuGENE HD reagent. 24 h after transfection, the cells were trypsinized and seeded (subconfluent) in a P6 dish with a glass coverslip. 48 h after transfection, the cells were fixed for 15 min at room temperature (dark) in 4% PFA/PBS. Afterward, they were washed three times in 1× PBS. For permeabilizing the cells, the coverslips were incubated for 25 min at room temperature (dark) in 0.25% Triton X-100/PBS. Afterward, the cells were washed three times in 1× PBS and blocked for 1.5 h at room temperature (dark) with 2% BSA/PBS. For the incubation with the first (1.5 h) and secondary antibody (1 h), 2% BSA/PBS solutions with the respective antibody dilutions were prepared. Three washing steps with 1× PBS preceded and followed the second antibody step, respectively. Finally, the cells were stained with 50 ng/ml DAPI/PBS for 2 min at room temperature, followed by three washings steps in 1× PBS. The coverslips were mounted in 50% glycerol/PBS, and pictures were taken with a 63× oil objective at the fluorescence microscope Axiosvert 200M (Carl Zeiss AG) with the following (filter) settings: DAPI with G 365 (exc) FT 395 (beam splitter) and BP 445/50 (em) (filter set 49); Alexa 488 with BP 500/25 DMR 25 (exc), FT515 HE (beam splitter), BP 535/30 DMR 25 (em) [filter set 46 HE]; mCherry with BP 546/12 (exc), FT 560 (beam splitter), BP 575–640 (em); DIC III Nomarsky (using Axiovision 4.8 software).

Western blotting

For analyzing the exogenous expression of human BRG1 and its Q and I motif mutants on protein level, 500,000 H1299 cells or SW-13 cells were seeded 1 day before the transfection in a P10 dish. The cells were transfected according to the manufacturer’s protocol of the FuGENE HD reagent. 24 h after transfection, the transfected H1299 cells were trypsinized and completely transferred into two P150 dishes. 48 h after transfection, the cells were harvested by scraping in 1× PBS. In the case of the transfected SW-13 cells, the cells were trypsinized 24 h after transfection and seeded at a density of 2.5 million cells per P10 and selected with G418 (500 – 800 μg/ml) for 9 days (start point of selection is 72 h after transfection). SW-13 cells were harvested as described for the transfected H1299 cells. For analysis of endogenous expression of BRG1 in nontreated H1299 cells, the cells were harvested as described above.

After the harvesting procedure, the cells were pelleted by centrifuging them at 1600 × g for 15 min at 4 °C, and the pellets were washed once in 1× PBS. The cells were finally centrifuged at 4 °C at 1600 × g for 10 min, and the cell pellets were frozen in liquid nitrogen for their final storage at −80 °C. For the lysis
procedure, one cell pellet was dissolved in a volume (varying from 50 to 200 μl) appropriate to the pellet size in 20 mM Tris, pH 7.6, 150 mM KCl, 1.5 mM MgCl₂, 0.5 mM EGTA, 0.1% IGEPA-L-CA630, 10% glycerol, 1× proteinase inhibitor mixture and sonified in a water bath supplemented with crushed ice for 5 min at level “high” with 30 s on and off. The cell lysates were pelleted by centrifugation at 4 °C for 15 min at 16,100 × g. According to Bradford measurements, 10–50 μg of the supernatants (whole cell extract) were supplemented with Laemmli buffer and loaded on 6.5% SDS gels, which were transferred to PVDF membranes via semidry blotting procedure. The Western blots were performed as described in Ref. 49 with the respective antibodies and analyzed at a LAS-3000 reader (Fuji film).

**Cell sorting for impedance measurements (ECIS) (see below)**

One day before transfection, 1.34 × 10⁶ cells were seeded in a P150 plate. 24 h later, the cells were transfected according to Ca₃(PO₄)₂ method (52). Briefly, 58.50 μg of (plasmid) DNA were mixed with 860 μl of autoclaved and sterile filtrated (0.1 μm) H₂O. Subsequently, 112 μl of 2 μM CaCl₂ were applied to the mixture. Finally, 1 ml of 2× HEPS buffer (0.274 mM NaCl, 1.5 mM Na₂HPO₄, 54.6 mM HEPS, pH 7.1) had to be subjoined dropwise. The whole transfection mixture was inverted three times and incubated for 1–15 min at room temperature before being added (2 ml in total per P150) to the cells, supplemented in the meantime with 20 ml of medium with fetal calf serum. In case of insufficient transfection efficiencies, the cells were treated again with the same transfection mixture the next day.

48 h after the first round of transfection, the cells were harvested by trypsinization and centrifuged at 500 × g for 5 min at room temperature. The cells were then resuspended in FACS buffer (1× PBS (Ca²⁺ and Mg²⁺ free), 25 mM HEPS, 2 mM EDTA, 2% dialyzed fetal calf serum, 50 μg/ml DNase I) at a density of 20–80 × 10⁶ cells/ml. The cells were kept on ice until use and filtered through a 30-μm preseparation filter into polypropylene tubes, shortly before sorting on a FACSDia I lu high-speed cell sorter (BD, Heidelberg, Germany). A 488-nm laser and a 610/20 bandpass filter were applied and the flow rate was between 500 and 5000 events/s. An 85-μm nozzle was used, and the fluid pressure was set to 45 p.s.i. Nontransfected cells were used for threshold definition. To minimize cell loss after the sorting procedure, mCherry-positive cells were sorted into tubes filled with medium (supplemented with penicillin/streptomycin) and kept on ice until being seeded in 8W10E+ PET microelectrode arrays (see “Cell proliferation monitored by ECIS”).

**Cell proliferation monitored by ECIS**

Sorted H1299 cells were transferred from polypropylene tubes into 15-ml falcon tubes and centrifuged at 800 × g for 5 min at room temperature. Supernatant was discarded, and the cell pellet was resuspended with 500 μl of culture medium, supplemented with penicillin/streptomycin. The cells were counted with a “Neubauer” counting chamber, and finally the cell population was adjusted to a cell density of 18,750–37,500 cells/ml by adding culture medium (supplemented with penicillin/streptomycin). For ECIS measurements, (overnight) culture medium-equilibrated 8W10E+ PET (polyethylene terephthalate) microelectrode arrays from Applied BioPhysics (Troy, NY)—treated in advance for 30 s with an argon plasma to clean and sterilize the electrode surface (Harrick Plasma)—were used. Each array contains eight wells, which can be analyzed almost in parallel. Each well (A = 0.8 cm²) has two sets of 20 circular electrodes (Ø = 250 μm) located on interdigitated fingers. This electrode structure offers the opportunity to analyze the impedance integrated over the entire growth area, as useful for proliferation studies with only low cell densities present in the well at the beginning of the experiment. Each well of the ECIS electrode array was inoculated (subconfluent) with 7500 cells (equates to 200–400 μl of the adjusted cell density). After cells were added (final volume, 400 μl per well), the electrode array was covered with a polystyrene lid and had to be incubated under a laminar flow for further 15 min, followed by an incubation step of 3–4 h in a cell culture incubator at 37 °C and 5% CO₂, to facilitate the sedimentation process and achieve homogeneous surface coverage with cells.

Afterward, the arrays were analyzed for proper cell sedimentation and homogeneous distribution using a Diaphot microscope (Nikon) at a magnification of 4×. To avoid evaporation of the medium in general, sterile water was filled into the reservoirs between the wells of respective arrays. For electrical contact with the measurement system, the electrode arrays were mounted in an electrode holder placed inside a humidified incubator (37 °C, 5% CO₂), and the ECIS measurement was started using the commercially available device ECIS Z (Applied BioPhysics, Troy, NY). The system contains impedance analyzer, frequency generator, microcontroller, and relay in one component. The relay switch makes every well individually addressable for the impedance analyzer. The ECIS device is connected to a computer for data storage and recording and is operated by the commercially available ECIS software (version 1.2.169), which also enables data analysis. The measurement was performed in multi-frequency/time mode, recording the complex impedance as a function of frequency along a range of 62.5–64 kHz at 11 predetermined frequencies (62.5, 125, 250, 500, 1000, 2000, 4000, 8000, 16,000, 32,000, and 64,000 Hz). The time resolution in a multi-frequency/time experiment for two electrode arrays of eight wells each was determined to ~ 4 min.

Instead of using the complex impedance, the capacitance, C, at a sensitive AC frequency of 32 kHz was followed over time to monitor cell proliferation. For high frequencies (f > 10 kHz) for electrodes with 250-μm diameter, as used here), the current can capacitively couple through the plasma membranes (transcellular current pathway). It has been shown that the decrease in total capacitance is linearly correlated with the increase in electrode surface coverage with cells (47) what renders high-frequency capacitance readings the most direct parameter reporting on electrode surface coverage and, thus, cell proliferation as a function of time. After 7–9 days, the cells of almost all wells reached full confluency, and measurements were stopped. At the end of the measurements, pictures were made from each well with a Diaphot microscope (Nikon) at a magnification of 4×. The data were transferred to Excel, and normalized values of C, termed C_n, were plotted as a function of time (done in Excel). Capacitance data were normalized to the respective value of the electrode covered with attached and equilibrated cells in low density (~5–6 h after seeding). Normalized data
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Table 1
Start and end values of $C_{\text{norm}}$ from two independent proliferation experiments, taken for slope calculation

|                | vc_1       | wt_1       | K785R_1    | Q758A_1    | vc_2       | wt_2       | K785R_2    | Q758A_2    |
|----------------|------------|------------|------------|------------|------------|------------|------------|------------|
| $C_{\text{norm}}$ start | 0.91 ± 0.02 | 0.91 ± 0.01 | 0.86 ± 0.02 | 0.91 ± 0.01 | 0.92 ± 0.01 | 0.91 ± 0.01 | 0.891 ± 0.008 | 0.90 ± 0.02 |
| $C_{\text{norm}}$ end    | 0.30 ± 0.02 | 0.29 ± 0.02 | 0.302 ± 0.008 | 0.43 ± 0.02 | 0.31 ± 0.02 | 0.28 ± 0.01 | 0.36 ± 0.03 | 0.35 ± 0.04 |

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provide a better comparison between different wells or even between different experiments. The following two parameters were chosen for the quantitative evaluation of the capacitance time course data in Excel: (i) the time point of half-maximal capacitance decrease ($T_{1/2}$), which corresponds to half-maximal proliferation of the cells; and (ii) the slope of capacitance decrease (s) to further quantify the rate of proliferation. Table 1 shows the $C_{\text{norm}}$ values, between which the slope s of the curves was calculated using a linear fit. The data presented in Table 1 were obtained from two biological replicates with three or four technical replicates each. The $T_{1/2}$ values were obtained by extracting from the capacitance time course the time needed for half-maximal $C_{\text{norm}}$ decrease (calculated between initial and final baseline values).

Creating a BRG1 3D homology model via I-TASSER

For the ATPase domain of human BRG1 isoform 2 (P51532-2), a 3D homology model was computed by using the I-TASSER server with default parameters (53). I-TASSER generates for each model two scores, namely the C score and the TM score. The C score estimates the quality of the predicted model and typically ranges from $-5$ to $2$; reliable models possess positive values. The TM score measures the structural similarity of the model and the template and ranges from $0$ to $1$, where $1$ indicates perfect correspondence. The C score of the model was 1.57, and the TM score was $0.93 \pm 0.06$; thus, both values signal high model quality. The model was further optimized by running an energy minimization. The ligand ATP$\gamma$S was introduced by superposition with the structure of the ATPase domain of yeast CHD1 (Protein Data Bank code 3MWY). A relaxed structure was calculated by means of GROMACS (54) (version 5.1.2) and the AMBER03 (55) force field. The structure was placed in a rectangular water box, and the system was neutralized adding NaCl ions. The solvated system was energy minimized until reaching convergence. Subsequently, a two-part equilibration phase (each lasted 50 ps) was started, consisting of an NVT (constant particle number (N), volume (V), and temperature (T) is regulated via a thermostat) simulation followed by an NPT (constant particle number (N), temperature (T), and pressure (P) is regulated via a barostat) simulation (56).
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