

Plasma-membrane-anchored growth factor pro-amphiregulin binds A-type lamin and regulates global transcription

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Summary

Amphiregulin (AR), a member of the EGF family, is synthesized as a type I transmembrane protein precursor (proAR) and expressed on the cell surface. Shedding of proAR yields a transmembrane-cytoplasmic fragment (AR-CTF), as well as a soluble AR. Here we demonstrate that the proAR-shedding stimuli trigger endocytosis of both AR-CTF and un-shed proAR. ProAR translocates from the plasma membrane to the inner nuclear membrane, whereas AR-CTF is translocated to the lysosome via retrograde membrane trafficking. Nuclear envelope localization of proAR involves truncation of the C-terminus, which subsequently activates the ER-retrieval signal. The truncated form of proAR interacts with A-type lamin and is retained at the inner nuclear membrane. Heterochromatin

formation is then induced and global transcription is transiently suppressed. This study gives new insight into epigenetic chromatin organization in mammalian cells: a plasma-membrane-anchored growth factor is targeted to the inner nuclear membrane where it participates in dynamic chromatin organization and control of transcription.

Supplementary material available online at
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Introduction

Over the 25 years, research on the epidermal growth factor (EGF) family and EGF receptors has provided numerous key insights into development, homeostasis and disease. The EGF family comprises 13 members, all of which are synthesized as type I transmembrane protein precursors and are subsequently expressed on the plasma membrane (Higashiyama et al., 2008). These transmembrane forms serve not only as juxtacrine factors (Anklesaria et al., 1990; Higashiyama et al., 1995; Singh and Harris, 2005), but also as intermediaries of EGF-receptor (EGFR) transactivation (Blobel, 2005; Higashiyama et al., 2008), which can play a major role in signaling by G-protein-coupled receptors (GPCRs), cytokine receptors and receptor tyrosine kinases (Rozengurt, 2007). Transactivation of EGFRs is mediated, in many cases, by soluble forms of EGF-family ligands, which are cleaved from their membrane-anchored forms (proforms) in a process termed 'ectodomain shedding' (Higashiyama et al., 2008).

The precursor of amphiregulin (proAR), which is a member of the EGF family, shares overall structural homology with other members of the EGF family. ProAR shedding from the cell surface involves a disintegrin and metalloprotease 17 (ADAM17) (also known as tumor necrosis factor- α converting enzyme or TACE) (Sahin et al., 2004; Hinkle et al., 2004). Ectodomain shedding of proAR is induced by stimulants, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Activation of proAR shedding produces a plasma-membrane-anchored remnant C-terminal fragment (AR-CTF) and leaves a significant amount of

un-shed proAR on the cell surface (Tokumaru et al., 2000). The fate of AR-CTF and proAR post-activation of the shedding process remains unclear. Recently we reported that ectodomain shedding of proHB-EGF, another member of the EGF family, evokes two independent signaling pathways: EGFR signaling induced by the shed extracellular domain and signaling driven by a remnant peptide comprised of the transmembrane and cytoplasmic domains (Nanba et al., 2003). Cytoplasmic domain signaling is mediated by interaction with at least two transcriptional repressors, known as promyelocytic leukemia zinc finger (PLZF) and B-cell lymphoma 6 (Bcl6), reversing PLZF- and Bcl6-mediated gene repression (Nanba et al., 2003; Kinugasa et al., 2007). We have also demonstrated translocation of proHB-EGF from the cell surface to the inner nuclear membrane (INM), where binding to the transcriptional repressors probably occurs (Hieda et al., 2008).

The nuclear envelope consists of the INM and an outer nuclear membrane (ONM), which are joined at the nuclear pore membrane. The ONM is structurally continuous with the peripheral ER. The nuclear lamina is a scaffold-like network of protein filaments underneath the INM. The scaffold consists primarily of type V intermediate filament proteins: lamin A/C, which is encoded by a single gene (*LMNA*) and expressed only in differentiated cells; and lamin B, which is encoded by two human genes (*LMNB1* and *LMNB2*) and found in nearly all somatic cells (Vlcek et al., 2001; Gruenbaum et al., 2005). Lamins support a broad range of functions through interaction with

various proteins, including INM proteins, chromatin, components of the RNA-polymerase-II-dependent transcription complex, and DNA replication complexes, suggesting that lamins are involved not only in nuclear structures, but also in DNA replication and gene expression (Spann et al., 1997; Spann et al., 2002; Goldman et al., 2002; Schirmer et al., 2003). Furthermore, it has been reported that mutations in *LMNA* or in the A-type lamin-binding proteins cause various diseases termed laminopathies. To date, there are about 50 known mutations in *LMNA* that cause laminopathies. These diseases affect striated muscle (Emery-Dreifuss muscular dystrophy or EDMD), cardiac muscle (dilated

cardiomyopathy CMD1A), limb-girdle muscle and neurons (Charcot-Marie-Tooth disorder AR-CMT2B1), adipose tissue (familial partial lipodystrophy FPLD), and adipocytes and bone (mandibuloacral dysplasia MAD) (Mounkes et al., 2003).

The results of the present study demonstrate that (1) AR-CTF and un-shed proAR are internalized in response to ectodomain-shedding stimuli and then translocated to lysosomes and ER or nuclear envelope, respectively; (2) un-shed proAR is subsequently translocated to the INM where it is retained; and (3) the INM-targeted un-shed proAR induces heterochromatin formation and suppression of global transcription.

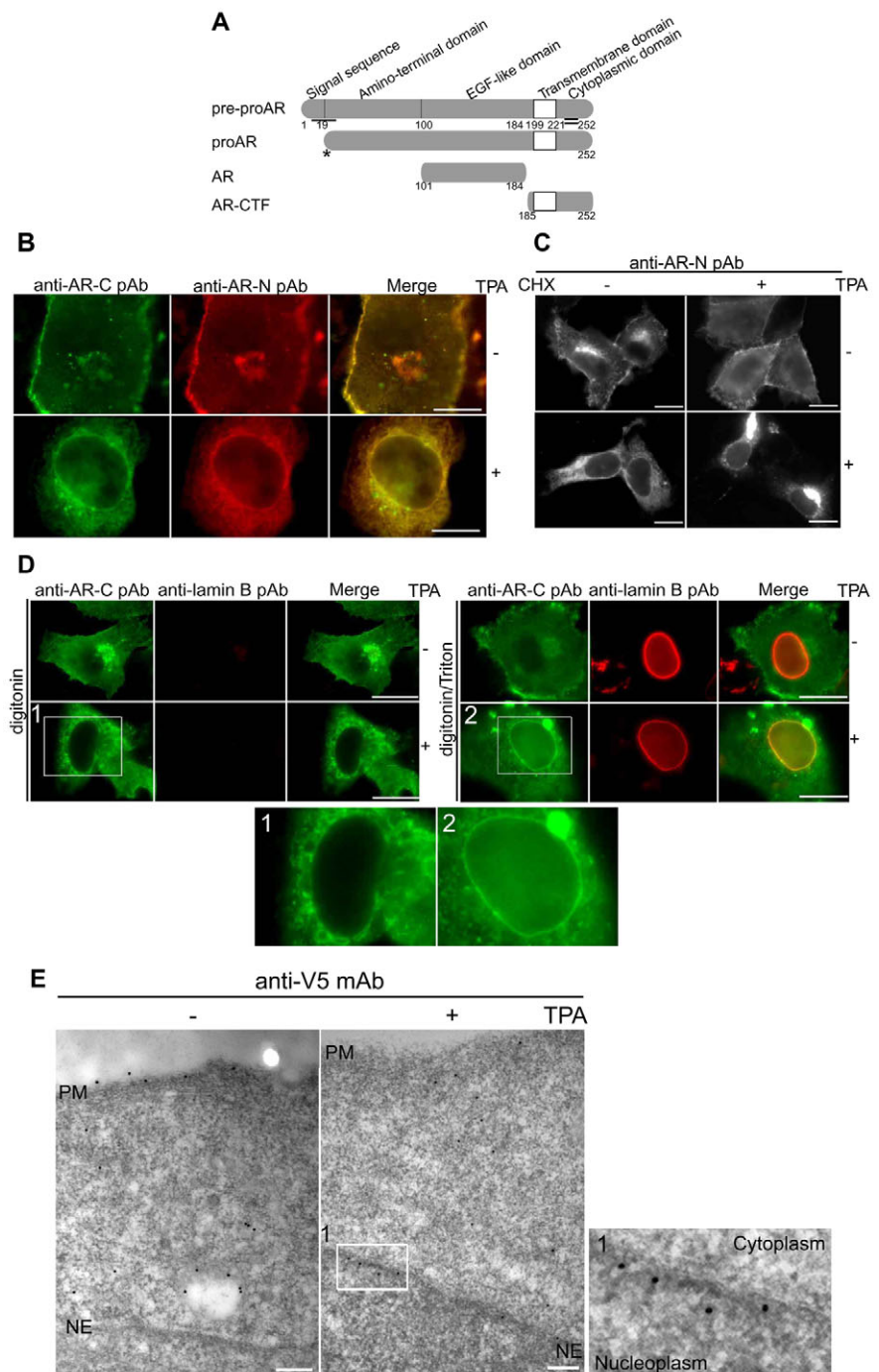


Fig. 1. ProAR translocates from the plasma membrane to the inner nuclear membrane. (A) Schematic representation of human *AREG* gene products. The AR coding region is translated as a precursor form (pre-proAR) with five structural domains consisting of predicted 252 amino acids (Plowman et al., 1990). The signal sequence is trimmed off, and the resulting protein is expressed on the plasma membrane as proAR whose N-terminal sequence is not yet determined (*). In response to various stimuli, proAR is shed at the juxtamembrane domain, resulting in the production of AR and AR-CTF. Anti-AR-N and anti-AR-C pAbs specifically recognize the extracellular and the cytoplasmic domains of proAR, respectively. The single line is the epitope region of anti-AR-N pAb, and double line is that of anti-AR-C pAb. (B) HeLa cells were transfected with a full-length *AREG* plasmid, then incubated with or without TPA. Cells were immunostained with anti-AR-N or anti-AR-C pAb. (C) HeLa cells transiently expressing *AREG* were incubated with 20 $\mu\text{g}/\text{ml}$ cycloheximide for 60 minutes. The cells were then treated with TPA and immunostained with anti-AR-N pAb. (D) HeLa cells transiently expressing *AREG* were permeabilized with digitonin and then fixed. The cells were re-permeabilized with (left panels) or without (right panels) Triton X-100, and immunostained with anti-AR-C (green) or/and anti-lamin B (red) pAbs. The lower panels are high magnification images of the boxed regions labelled 1 and 2. Scale bars: 5 μm . (E) Ultra-thin sections were stained with anti-V5 mAb and 15 nm gold-conjugated secondary antibodies. Right panel, without TPA; middle panel, with TPA; left panel, high magnification image of boxed region in middle panel. PM, plasma membrane; NE, nuclear envelope. Scale bars: 200 nm.

Results

Un-shed proAR and AR-CTF are targeted to the INM and lysosome, respectively

Amphiregulin (AR; official symbol AREG) is synthesized as a type I transmembrane protein (pre-proAR) and is expressed on the plasma membrane as a 25-50 kDa precursor (proAR). ProAR contains an extracellular EGF-like domain, a transmembrane segment and a short cytoplasmic tail (Fig. 1A). ProAR is cleaved at the juxtamembrane domain via metalloprotease activation, yielding a soluble AR and a C-terminal fragment that contains transmembrane and cytoplasmic segments (AR-CTF). HeLa cells transiently transfected with *AREG* were immunostained with two polyclonal antibodies, anti-AR-N and anti-AR-C, which recognize the extracellular and cytoplasmic domains of proAR, respectively. ProAR-positive staining was visualized at the plasma membrane and the perinuclear organelles under steady-state conditions (Fig. 1B, upper panels). The perinuclear signal colocalized with the Golgi complex marker protein, GM130 (data not shown). In the presence of a shedding stimulus (TPA), the anti-AR-N and anti-AR-C signals were both observed in the reticular meshwork, evidently in the ER and nuclear envelope (Fig. 1B, lower). This result suggests translocation of un-shed proAR from the plasma membrane to the

ER or nuclear envelope. To verify that the proAR in the reticular meshwork was not newly synthesized protein, de novo synthesis was blocked with cycloheximide. In the presence of cycloheximide, the proAR signal in the Golgi disappeared; however, proAR staining was evident at the plasma membrane and shifted to the ER and nuclear envelope after TPA stimulation (Fig. 1C). Thus, proAR on the plasma membrane translocates to the ER and nuclear envelope.

If proAR is targeted to the INM, cytoplasmic domains face the nucleoplasm and can physically interact with proteins localized inside the nucleus. Thus, it is crucial to determine whether nuclear-envelope-targeted proAR localizes to the ONM or to the INM. We took advantage of the fact that low concentrations of digitonin selectively permeabilize the plasma membrane, but leave the nuclear membrane intact (Adam et al., 1990). Digitonin treatment allowed detection of anti-AR-C signal at the plasma membrane and Golgi without TPA treatment and at the ER after TPA treatment. Lamin B signal was not detected, whether cells were treated with TPA or not. Lamins form a network of filaments underlying the INM and require Triton X-100 permeabilization for antibody detection. After Triton X-100 treatment and digitonin permeabilization, an AR-C signal was detected at the nuclear

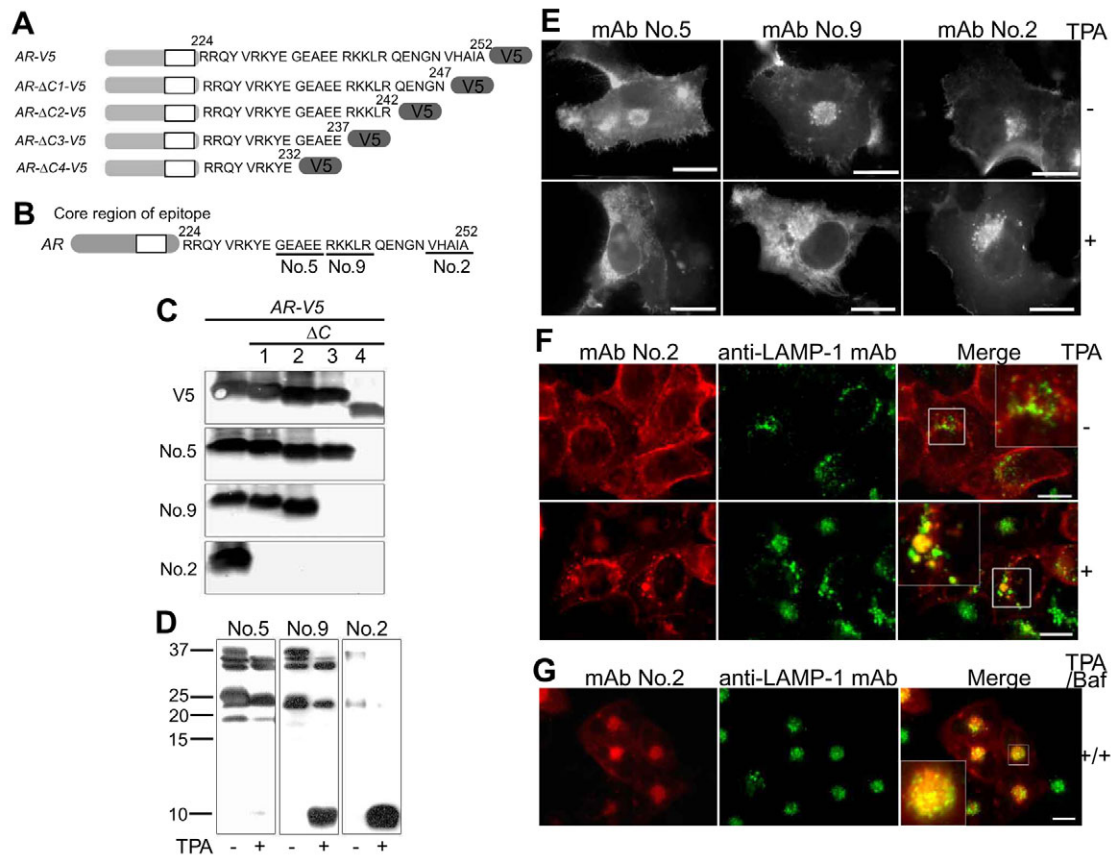


Fig. 2. ProAR is targeted to the nuclear envelope. (A) Schematic presentation of AR deletion mutants. All constructs (AR-V5, AR- Δ C1-V5, AR- Δ C2-V5, AR- Δ C3-V5 and AR- Δ C4-V5) were V5-tagged at the C-terminus. (B) Three monoclonal antibodies mAb2, mAb5 and mAb9 against the cytoplasmic domain of proAR were established. The core epitope region for each antibody is underlined. (C) Total lysates of HeLa cells expressing AR-V5 or AR- Δ C-V5 mutants were analyzed by immunoblotting with the three mAbs. An anti-V5 mAb was used as a positive control. (D) Lysates of HeLa cells expressing wild-type *AREG* were immunoblotted with anti-AR-C mAbs. (E and F) HeLa cells transiently expressing wild-type *AREG* were incubated with or without TPA for 30 minutes and immunostained with the anti-AR-C mAbs (E) or mAb2 and anti-LAMP-1 mAbs (F). (G) HeLa cells transiently expressing wild-type *AREG* were treated with 50 nM Bafilomycin (Baf) for 60 minutes and incubated with TPA in the presence of Baf. The cells were then immunostained with mAb2 and anti-LAMP-1 mAb. Scale bars: 5 μ m.

envelope in response to TPA treatment and a lamin B signal was evident, which was independent of TPA treatment (Fig. 1D). The detection of AR-C signal after Triton X-100 and digitonin treatment indicated that the cytoplasmic domain of proAR was exposed into the nucleoplasm after TPA stimulation (Fig. 1D, lower panel). These results suggest that the un-shed proAR could be targeted to the INM after TPA treatment. Next, we detected proAR at the nucleoplasmic face of the nuclear envelope in TPA-treated cells using immunoelectron microscopy (Fig. 1E). We conclude that un-shed proAR can localize at the INM upon shedding stimuli.

To identify the functional domain(s) involved in intracellular localization, we prepared monoclonal antibodies (mAbs) against the cytoplasmic domain of proAR, and obtained three clones (termed mAb2, mAb5 and mAb9) that recognized distinct epitopes (Fig. 2B). To identify the epitope region, we used the C-terminal deletion mutants of AR (Fig. 2A). The ability of mAb2, mAb9 and mAb5 to bind proAR was abrogated by deleting the C-terminal 5, 15 and 20 amino acids of proAR, respectively (Fig. 2C). In western blot analysis, mAb5 efficiently recognized proAR, but not the ~10 kDa AR-CTF (Fig. 2D). The mAb9 bound both proAR and AR-CTF, whereas mAb2 preferentially bound AR-CTF (Fig. 2D). Because AR-CTF encompasses the mAb5 epitope region, these results suggest that the mAb5 epitope region might be modified in AR-CTF. Immunofluorescent microscopy revealed that all mAbs stained the plasma membrane and the Golgi complex in the absence of TPA, although the signal from mAb2 was weaker than that from the other antibodies (Fig. 2E, upper panel). After TPA treatment, mAb5 and mAb9 detected proAR localized in the ER and nuclear envelope (Fig. 2E, lower panel), which was also detected with anti-AR-N pAbs (data not shown). However, mAb2 did not detect proAR in the ER and nuclear envelope (Fig. 2E, lower panel), suggesting that the mAb2 epitope might be masked or removed in proAR localized in this region. Interestingly, organelles detected by mAb2 were partially recognized by a mAb that was specific for LAMP-1, which is a lysosome marker protein (Fig. 2F), but were not recognized by anti-AR-N pAb (data not shown). In the presence of bafilomycin (Baf), which inhibits the lysosomal proton pump, thereby suppressing membrane trafficking through lysosomes, the signal for mAb2 significantly colocalized with the LAMP-1 signal (Fig. 2G).

These results imply that un-shed proAR targeted to the ER and nuclear envelope might be modified at the C-terminus (i.e. in the

mAb2 epitope region), whereas AR-CTF accumulates in lysosomes. We speculate that masking of the epitope recognized by mAb5 in AR-CTF might signal lysosome targeting. Thus, we focused hereafter on the localization and function of un-shed proAR.

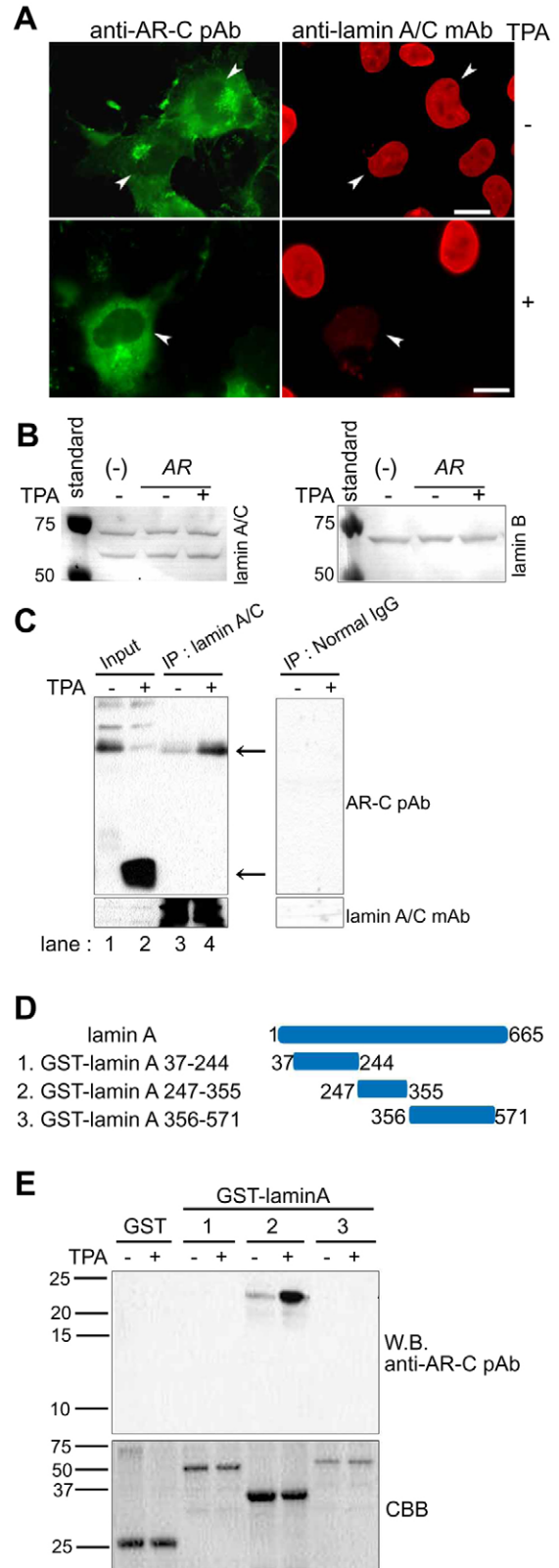


Fig. 3. ProAR interacts with A-type lamin. (A) HeLa cells transiently expressing wild-type *AREG* were incubated with or without TPA. Cells were stained with anti-AR-C pAb and anti-lamin A/C mAb. Arrowheads indicate *AREG*-expressing cells. Scale bars: 5 μ m. (B) Lysates of HeLa cells transiently transfected with or without wild-type *AREG* and treated with TPA for 60 minutes, were subjected to immunoblotting using anti-lamin A/C mAb (left) or anti-lamin B pAb (right). The left lanes show standard proteins. (C) HeLa cells transiently expressing wild-type *AREG* were incubated with or without TPA for 60 minutes. The cell lysates were subjected to immunoblotting with an anti-AR-C pAb (lane 1 and lane 2). The cell lysates were immunoprecipitated with an anti-lamin A/C mAb or normal mouse IgG, followed by western blotting using anti-AR-C pAb and anti-lamin A/C mAb. Arrows indicate the molecular mass of lamin A/C-interacted (upper) and AR-CTF (lower). (D) Schematic representation of GST-fused lamin A deletion mutants. (E) The cell lysates from cells transiently expressing wild-type *AREG* were incubated with GST or GST-lamin A derivatives, which were pulled down by glutathione-Sepharose beads. Bound proteins were analyzed by western blotting using the anti-AR-C pAb (upper panel). GST fusion proteins were analyzed by SDS-PAGE and stained with CBB (lower panel).

Un-shed proAR interacts with A-type lamin

Some proteins are tethered to the INM because of interactions with INM-resident proteins (Holmer and Worman, 2001). The intensity of anti-lamin A/C mAb staining decreased in AR-expressing cells after TPA treatment (Fig. 3A), even though lamin protein levels remained unchanged (Fig. 3B). This observation suggests a physical interaction between AR and A-type lamin. Immunoprecipitation was used to examine this possibility. Un-shed proAR, but not AR-CTF, co-precipitated with an anti-lamin A/C mAb; TPA treatment markedly enhanced binding between AR and lamin A/C (Fig. 3C). Precipitated un-shed proAR migrated slightly faster than proAR, suggesting that it was smaller than proAR (Fig. 3C). Neither normal mouse IgG nor anti-lamin B pAb immunoprecipitated un-shed proAR (Fig. 3C and supplementary material Fig. S1A). GST-tagged deletion mutants of lamin A pulled down proAR from the total cell lysate. This result demonstrates that a sequence within the lamin A

region spanning residues 247-355 (shared with lamin C) is essential for the interaction with AR (Fig. 3D,E).

Next, siRNA duplexes targeting lamin A/C were used to examine the lamin A/C requirement for INM localization of un-shed proAR. Transfection with siRNA duplexes resulted in a marked and specific reduction in levels of lamin A/C protein (Fig. 4A). Lamin A/C downregulation abrogated localization of un-shed proAR in the nuclear envelope, but did not affect accumulation of proAR in the perinuclear structure in the presence of TPA (Fig. 4B). Control siRNA or knockdown of lamin B did not affect nuclear envelope targeting of proAR (Fig. 4B and supplementary material Fig. S1B). Thus, it was concluded that lamin A/C is essential for localization of proAR to the nuclear envelope.

Un-shed proAR that interacts with A-type lamin is truncated at the C-terminus

Because the proAR that interacted with lamin A/C migrated slightly faster than proAR (Fig. 3C) and mAb2 did not recognize the proAR targeted to the ER and nuclear envelope in immunofluorescence studies (Fig. 2E), we hypothesized that the epitope recognized by mAb2 might be removed from INM-localized proAR. This hypothesis was tested by immunoprecipitation studies. As shown in Fig. 5A, mAb2 was unable to detect proAR that had been co-

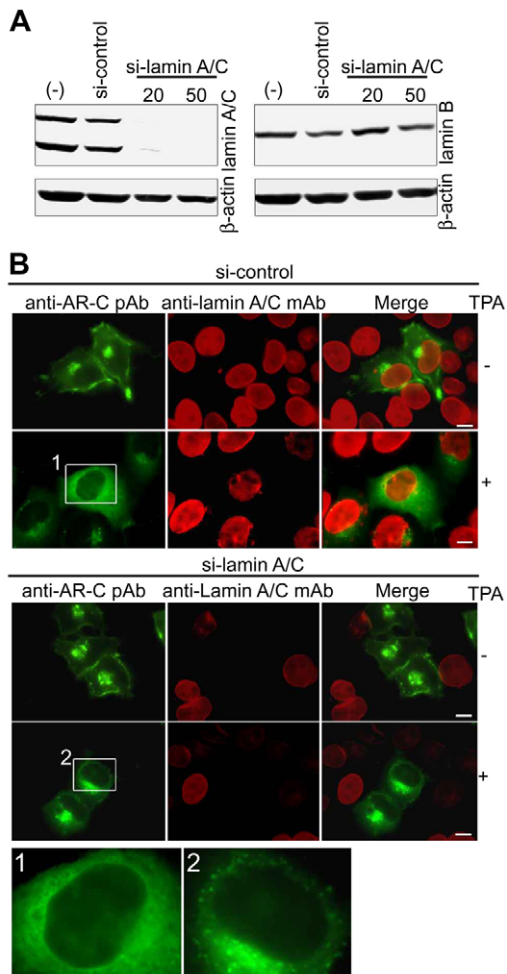


Fig. 4. A-type lamin is essential for targeting of proAR to the nuclear envelope. (A) HeLa cells were transfected with 20 nM or 50 nM of lamin A/C-targeting duplex siRNA (si-lamin A/C) or control siRNA (si-control). After 72 hours, the cells lysates were analyzed by western blotting with anti-lamin A/C mAb or anti-lamin B pAb. (B) HeLa cells were transfected twice: first with 20 nM of control siRNA (upper) or lamin A/C-siRNA (lower); and, after 48 hours, with wild-type *AREG* plasmid. After 24 hours, cells were stimulated with or without TPA, and then fixed. Cells were stained with anti-AR-C pAb and anti-lamin A/C mAb. The lower panels (1 and 2) are high magnification images of the boxed areas above. Scale bars: 5 μ m.

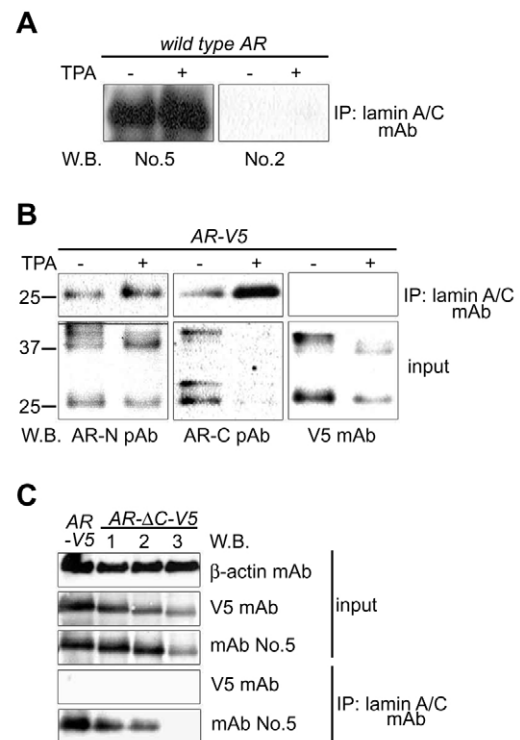


Fig. 5. Un-shed proAR that interacts with A-type lamin is truncated at the C-terminus. (A) HeLa cells transiently expressing wild type *AREG* were incubated with or without TPA. Cell lysates were immunoprecipitated with anti-lamin A/C mAb and analyzed by western blot using the mAb5 and mAb2. (B) HeLa cells transiently expressing AR-V5 were incubated with or without TPA. Cell lysates were immunoprecipitated with anti-lamin A/C mAb. Cell lysates (upper) and precipitated proteins (lower) were analyzed with indicated mAbs. (C) Cell lysates of HeLa cells expressing AR-V5, AR- Δ C1-V5 (lane 1), AR- Δ C2-V5 (lane 2) or AR- Δ C3-V5 (lane 3) were immunoprecipitated with anti-lamin A/C mAb. Cell lysates and precipitated proteins were analyzed with β -actin, anti-V5 mAb or mAb5.

precipitated with lamin A/C. This result implies that proAR that interacts with lamin A/C lacks the portion of the C-terminus containing the epitope recognized by mAb2. We examined this possibility in greater detail using C-terminal-V5-tagged proAR (Fig. 2A). Anti-AR-N and anti-AR-C pAbs recognized the co-precipitated proAR, but an anti-V5 mAb did not (Fig. 5B). These results demonstrate that the proAR that interacts with lamin A/C did not contain the V5 tag. Furthermore, two deletion mutants, AR- Δ C1-V5 and AR- Δ C2-V5 (Fig. 2A) were co-precipitated with an anti-lamin A/C mAb. Thus, the C-terminal 10 amino acids are not required for lamin A/C binding (Fig. 5C). All proAR proteins co-immunoprecipitated with anti-lamin A/C mAb were the same size (Fig. 5C, bottom panel) and were not detected using an anti-V5 mAb (Fig. 5C, second panel from the bottom). Taken together, these results show that proAR interacting with lamin A/C is truncated at the C-terminus. Mass spectrometric analysis of proAR co-immunoprecipitated with anti-lamin A/C mAb was performed to determine the cleavage site. However, the lysine- and arginine-rich sequence in the cytoplasmic domain made it difficult to identify the enzymatic digest products.

Lys239 and Lys240 in the cytoplasmic domain of proAR are essential for targeting to the nuclear envelope

Most ER-resident type I transmembrane proteins have the ER-retention signal, K-K-x-x (x is any amino acid), at the C-terminus

(Nilsson et al., 1989; Jackson et al., 1990). Interestingly, the K-K-x-x motif exists in the cytoplasmic domain of proAR, although it is not exposed at the distal terminus (Fig. 6A). When Ala residues were substituted for Lys239 and Lys240 (AR-m2) in proAR, targeting to the nuclear envelope did not occur, even after TPA treatment. Instead, the proAR-m2 accumulated in the perinuclear region colocalized with TGN46, a trans-Golgi marker (Fig. 6B). These results indicate that Lys239 and/or Lys240 are essential for ER targeting. To analyze the mechanism of proAR targeting to the ER, a V5 epitope tag was inserted into proAR just downstream from the transmembrane domains (AR-V5-C). The V5-tag insertion had no effect on localization of proAR to the plasma membrane and Golgi under steady state conditions. Likewise, insertion of the V5 tag did not affect targeting to the nuclear envelope in response to TPA stimulation (Fig. 6C,D). Notably, deletion of 10 residues at the C-terminus (AR-V5- Δ C10) to expose ²³⁹KKLR₂₄₂-induced localization of proAR to the ER and nuclear envelope, even in the absence of TPA treatment (Fig. 6D). However anti-lamin A/C mAb staining was only slightly reduced in AR-V5- Δ C10 transfectants (compare Fig. 6E and Fig. 3A) and the interaction with lamin A/C was very weak (Fig. 6F). We then created an 11-residue deletion mutant (AR-V5- Δ C11), which exposed ²³⁸RKKL₂₄₁ at the C-terminus, and observed ER and nuclear envelope localization in the steady state. Anti-lamin A/C mAb staining was markedly reduced and a significant interaction with lamin A/C was observed (Fig. 6E,F). In addition, a 12-residue deletion mutant of

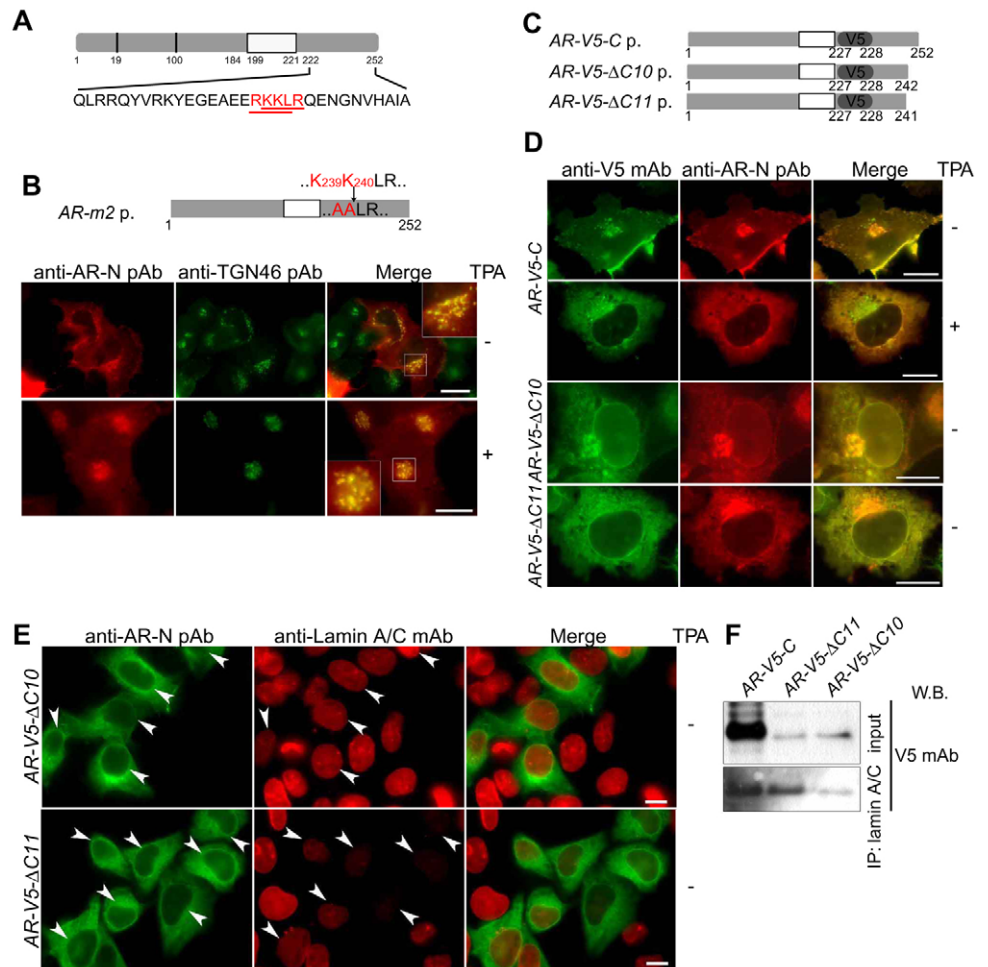


Fig. 6. Lysine239 and/or lysine240 in the cytoplasmic domain of proAR are essential for targeting to the nuclear envelope.

(A) Amino acid sequence of the cytoplasmic domain of proAR. (B) Schematic representation of mutations in K239 and K240 in the cytoplasmic domain (AR-m2). AR-m2 p indicates AR-m2 precursor protein. HeLa cells transfected with AR-m2 were incubated with or without TPA and immunostained with anti-AR-N pAb (red) and anti-TGN46 pAb (green). (C) Schematic representation of V5-tag inserted AR and its deletion mutants. AR-V5-C p, AR-V5- Δ C10 p and AR-V5- Δ C11 p indicate precursor proteins. (D,E) HeLa cells transiently expressing AR-V5-C, AR-V5- Δ C10 or AR-V5- Δ C11 were incubated with or without TPA, then immunostained with anti-V5 mAb and anti-AR-N pAb (D) or with anti-AR-N pAb and anti-lamin A/C mAb (E). Arrowheads indicate AR-expressing cells. (F) Cell lysates of HeLa cells expressing AR-V5-C, AR-V5- Δ C10 or AR-V5- Δ C11 were immunoprecipitated with anti-lamin A/C mAb. Cell lysates (upper) and precipitated proteins (lower) were analyzed with anti-V5 mAb. Scale bars: 5 μ m.

proAR (AR-V5- Δ C12), in which ²³⁷ERKK₂₄₀ was exposed at the C-terminus, did not localize to the ER and nuclear envelope (data not shown). These results indicate that ²³⁹KKLR₂₄₂ and ²³⁸RKKL₂₄₁ in the cytoplasmic domain of proAR function as ER-retention signals when exposed at the distal C-terminus, but the activity of AR-V5- Δ C11 seems to be equivalent to that of the wild type, unlike AR-V5- Δ C10. Thus, the exact cleavage site remains to be identified. Although it is plausible that lamin-A/C-interacting proAR might lack 11 amino acids at the C-terminus, resulting in ER and nuclear envelope targeting.

Nuclear-envelope-targeted proAR masks a lamin A/C epitope in dense heterochromatin

The intensity of lamin A/C mAb staining was decreased in cells expressing nuclear-envelope-targeted proAR, even though A-type lamin protein levels remained unchanged (Fig. 3A,B) and anti-lamin

A/C pAb stained these cells (data not shown). Epitope masking of lamin A/C in immunofluorescence studies as a result of interactions with chromatin and other proteins is a well known phenomenon (Hozák et al., 1995; Dyer et al., 1997; Kumaran et al., 2002; Markiewicz et al., 2005). To explore epitope masking due to lamin interactions with chromatin or other proteins, AR-expressing cells were treated with DNase I, NaCl and/or Triton X-100 before fixation (Markiewicz et al., 2005). Triton X-100 and/or NaCl extraction had no effect on lamin A/C staining. When cells were treated with Triton X-100, NaCl and DNase I, the lamin A/C signal was apparently recovered (Fig. 7A). We tried to extract DNA using NaCl and DNase I; however, the efficiency of chromosomal DNA extraction, judged by Hoechst 33342 staining, was too low (data not shown). These results suggest that epitope masking occurs because of physical associations between lamin and chromatin. Intriguingly, the epitope for the anti-lamin A/C mAb is located in a region spanning amino residues 356-571 (data not shown), which corresponds to the chromatin-binding domain (Zastrow et al., 2004).

Nuclear-envelope-targeted proAR induces diverse heterochromatin assembly

To verify the physiological role of nuclear-envelope-targeted AR, we focused on the proAR-lamin-A/C interaction. We observed that nuclear envelope targeting of proAR induced heterochromatinization, as evidenced by strong staining with Hoechst 33342 (Fig. 7B). Overexpression of other INM proteins (e.g. HB-EGF and lap2 β) did not induce this heterochromatinization (supplementary material Fig. S3). Higher-order assembly of chromatin is thought to be largely determined by post-translational methylation of histone tails at H3 Lys9 (H3K9), which is essential for localization of HP1. As expected, it could be observed that Hoechst 33342 staining was accompanied by an increase in trimethylation of H3K9 (H3K9me3) in cells with nuclear-envelope-targeted proAR. Moreover, the heterochromatin protein, HP1 β colocalized with Hoechst 33342 staining in these cells (Fig. 7B). Western blot analysis confirmed the relative increase of H3K9me3 in cells transiently expressing proAR after TPA treatment and cells expressing AR- Δ 11 without TPA treatment (Fig. 7C).

Nuclear-envelope-localizing proAR suppresses global transcription

Heterochromatin can propagate, and thereby influence, gene expression in a region-specific

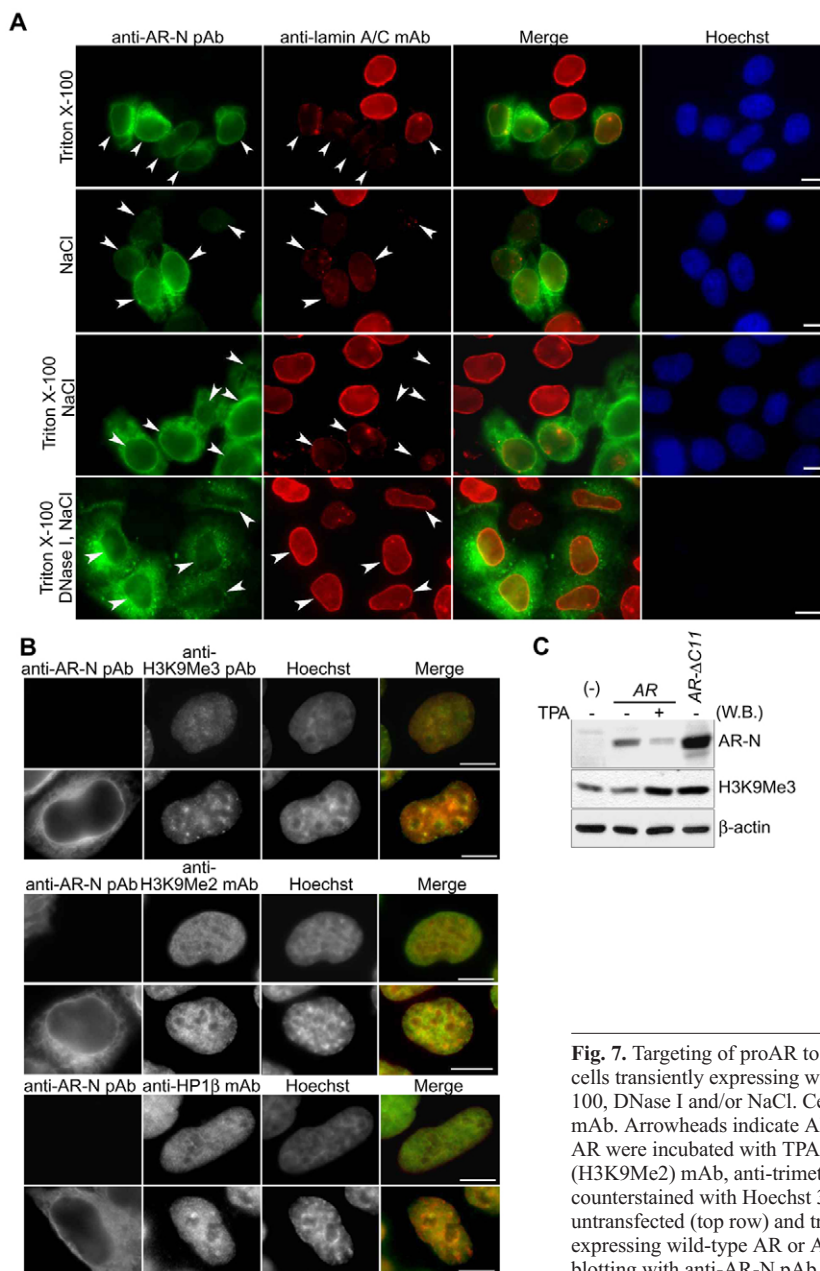


Fig. 7. Targeting of proAR to the nuclear envelope causes heterochromatin assembly. (A) HeLa cells transiently expressing wild-type AR were incubated with TPA and treated with Triton X-100, DNase I and/or NaCl. Cells were then stained with anti-AR-N pAb and anti-lamin A/C mAb. Arrowheads indicate AR-expressing cells. (B) HeLa cells transiently expressing wild-type AR were incubated with TPA and then stained with anti-AR-N pAb, anti-dimethylated H3K9 (H3K9Me2) mAb, anti-trimethylated H3K9 (H3K9Me3) pAb and anti-HP1 β pAb. Cells were counterstained with Hoechst 33342. Each panel shows representative cells that are untransfected (top row) and transfected (bottom row). (C) Cell lysates of HeLa cells transiently expressing wild-type AR or AR- Δ C11 treated with or without TPA were analyzed by western blotting with anti-AR-N pAb, anti-H3K9Me3 mAb and anti- β -actin mAb. Scale bars: 5 μ m.

and sequence-independent manner. When heterochromatin spreads across domains, it generally causes epigenetic repression of nearby sequences. Thus, the diverse heterochromatin formation

in AR-expressing cells prompted us to examine global transcriptional activity. Synthesis of RNA transcripts was monitored using incorporation of bromo-uridine (Br-U) (Iborra et al., 1998). This analogue does not affect activity of the major polymerizing enzyme RNA polymerase II. Newly synthesized Br-U-labeled RNA (Br-RNA), with the exception of nucleolar RNA, can be detected with anti-BrdU mAb after formaldehyde fixation (Koberna et al., 1999). In the present study, newly synthesized RNA was dramatically reduced in cells with nuclear-envelope-targeted proAR, even though Br-RNA intensity in untransfected cells was not affected by TPA treatment (Fig. 8A) and overexpressed HB-EGF or lap2 β (supplementary material Fig. S2B,C). To exclude the possibility that reduced transcriptional activity was due to cell death, cell proliferation and Br-U incorporation were monitored for 24 hours. Quantitative analysis showed that global transcription was dramatically suppressed after exposure to a shedding stimulus by approximately 80% for up to 4 hours, after which time transcription was recovered within 24 hours. Statistical analysis showed that there are significant differences between the presence and absence of shedding stimuli. Interestingly, ectopic expression of AR-V5- Δ C11, which is localized to the nuclear envelope in the absence of shedding stimuli suppressed global transcription without TPA treatment (Fig. 8C). Moreover the global transcription in the AR-V5-C-expressing cells in which lamin A/C was knocked down was not affected (supplementary material Fig. S3). These results indicate that proAR-lamin-A/C interaction induces heterochromatinization and global transcriptional repression. The total pool of hyperphosphorylated and hypophosphorylated largest RNA polymerase II subunit was unchanged in western blot analysis (data not shown). Heterochromatin assembly is commonly associated with large-scale chromatin condensation and reorganization of the nuclear domain. Both these conditions reduce the accessibility of transcription machinery to heterochromatic loci. Thus, it was concluded that nuclear-envelope-targeting of proAR suppressed the global transcription. This might be due to reduced accessibility rather than regulation of transcriptional machinery.

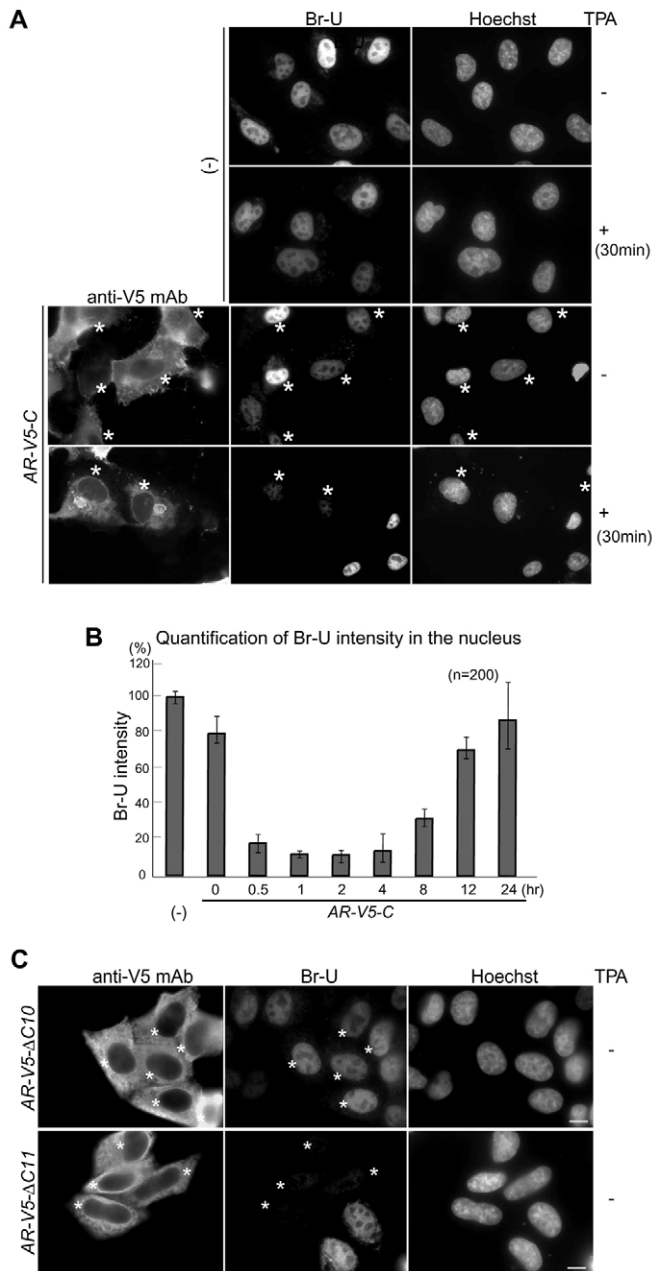


Fig. 8. Targeting of proAR to the nuclear envelope induces transient suppression of global transcription. (A,C) 5 mM Br-U was added in the presence or absence of TPA to the culture medium of HeLa cells transiently expressing AR-V5-C (A), AR-V5- Δ C10 or AR-V5- Δ C11 (C) followed by a 30 minute incubation. Cells were fixed and immunostained with anti-BrdU mAb, anti-V5 mAb and Hoechst 33342. Asterisks indicate AR-expressing cells. (B) HeLa cells transiently expressing AR-V5-C were incubated with or without TPA for 30 minutes and re-grown in fresh medium for the duration indicated. 5 mM Br-U was added to the culture medium 30 minutes prior to fixation. After fixation, Br-U incorporated in mRNA was stained with anti-BrdU mAb and fluorescent intensity in the AR-expressing cells was quantified ($n=200$). Statistical analysis was carried out as described in Materials and Methods. Scale bars: 5 μ m.

Discussion

A model of proAR targeting from the plasma membrane to the inner nuclear membrane

The efficiency of proAR-ectodomain shedding is moderate (Tokumaru et al., 2000), resulting in the production of AR, AR-CTF and un-shed proAR. Herein, we provide evidence that proAR is translocated from the plasma membrane to the INM, where it is tethered by interaction with A-type lamin. Based on our results, we propose a model for this retrograde transport pathway, as shown in Fig. 9. First, proAR is primarily localized at the plasma membrane. Shedding stimuli induce internalization of both AR-CTF and un-shed proAR. Internalized AR-CTF and un-shed proAR are then targeted to different destinations, the lysosome and INM, respectively, suggesting different functions. The molecular mechanisms of the differential intracellular sorting remain unknown, but two types of modifications in the cytoplasmic region may be key events: epitope masking and truncation of the C-terminus. Masking of the epitope recognized by mAb5 in AR-CTF was suggested by the observation that this antibody was barely able to detect AR-CTF, whereas mAb2 showed a strong signal. We speculate that this masking modification might act as a lysosomal-sorting signal. AR-CTF is likely to have a full-length cytoplasmic tail given the fact that it can bind to mAb2, even though

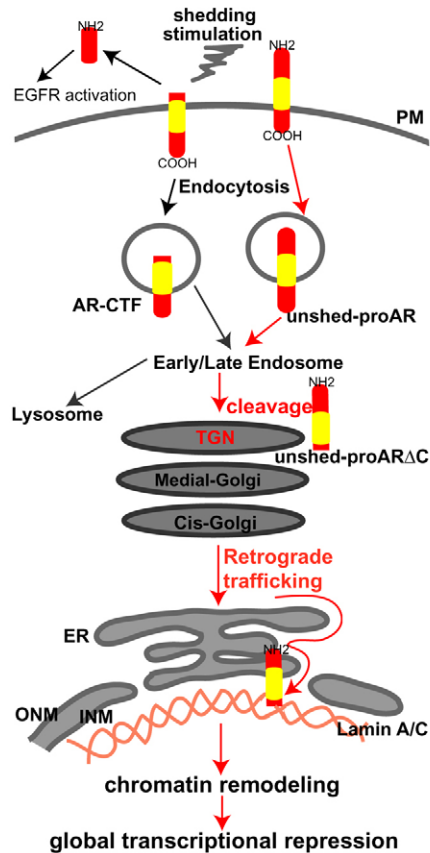


Fig. 9. Schematic representation of proAR intracellular trafficking. Without TPA stimulation, proAR primarily localizes at the plasma membrane. TPA induces partial proAR-ectodomain shedding, resulting in the production of a soluble growth factor (AR) and a C-terminal fragment (AR-CTF). In addition, TPA causes the endocytosis of both AR-CTF and the residual un-shed proAR. AR-CTF then translocates to the lysosome (black arrow) and un-shed proAR trafficks to the INM (red arrow). During translocation to the INM, proAR is truncated at the C-terminus, and targeted to the ER. Subsequently ER-localized proAR diffuses or is actively transported to the INM, where it is tethered via interaction with A-type lamin and downregulates global transcription. PM, plasma membrane.

INM-targeted proAR is truncated at the C-terminus. Deletion mutants of proAR suggest that the R/K-K-x-x sequence might function as an ER-retrieval signal. Disruption of this ER-retrieval-signal sequence in wild-type proAR (AR-m2) abrogated the INM targeting typically observed in response to shedding stimuli. Thus, proAR utilizes this ER retrieval signal to localize to the ER. Truncation of the cytoplasmic tail might be crucial in determination of the intracellular fate of AR-CTF compared with un-shed proAR. AR-V5- Δ C11 is localized at the nuclear envelope without shedding stimuli and decreased lamin A/C staining. AR-V5- Δ C11 is co-precipitated with anti-lamin-A/C antibody. AR-V5- Δ C10 is localized at the nuclear envelope in immunofluorescence studies; however, its effect on the lamin A/C staining and transcriptional repression is not relevant to cells expressing the wild-type proAR. Therefore we assume that removal of the 11 amino acids at the C-terminus of proAR is necessary to achieve nuclear targeting of proAR. This is the first report to describe exposure and activation of the internal ER-retrieval signal in a mammalian protein; it has been described for some protein toxins (e.g. *Pseudomonas*

exotoxin) (Sandvig and van Deurs, 2002). Accumulation of the AR-m2 mutant at the TGN suggests that proAR may translocate by way of the TGN, where truncation of the C-terminus might occur.

After translocation to the ER, proAR can diffuse laterally between the peripheral ER, ONM and INM via the nuclear pore complex, because here the cytoplasmic domain is small enough to pass through the aqueous channel. Alternatively, proAR can be translocated from the ONM to the INM via active transport, which requires protein interactions. Some integral INM proteins possess a basic-sequence motif that resembles a 'classical' nuclear localization signal (NLS) and binds to karyopherin- α (King et al., 2006). However, the cytoplasmic domain of proAR did not show any detectable NLS activity for soluble reporter proteins (data not shown).

Possible mechanism and roles for proAR-induced heterochromatin formation and transient suppression of global transcription

We observed that INM targeting of proAR triggers heterochromatin assembly and negatively regulates transcription by RNA polymerase II. Chromosomal DNA contains a high density of repetitive DNA elements, including constitutive heterochromatin. However, heterochromatin is also found at developmentally regulated loci, where the chromatin state can change in response to cellular signals and gene activity. This type of heterochromatin is known as facultative heterochromatin. The basic mechanisms that underlie proAR-induced heterochromatin formation are largely unclear. However, lamin-proAR interaction is likely to enhance the lamin-chromatin interaction because of the masking of an antigenic epitope in the chromatin-binding domain of lamin A/C. The ProAR-interacting domain in lamin A/C is shared with other lamin-A/C-binding proteins, such as the Kruppel/TFII-related zinc finger protein MOK2 (Dreuillet et al., 2002), retinoblastoma protein (Rb) (Ozaki et al., 1994) and LAP2 α (Markiewicz et al., 2005), suggesting that this might have an important role in their function. Rb and LAP2 α are well known regulators of cell cycle and gene transcription in the nucleoplasm. Thus, chromatin organization might be dynamically regulated via these soluble lamin-binding proteins throughout the nucleoplasm. Furthermore, the ability of heterochromatin to spread in *cis* and to be coordinately regulated with other heterochromatin regions in *trans* may play a role in diverse heterochromatin assembly. Interestingly, the disease of premature aging, Hutchinson-Gilford Progeria Syndrome (HGPS), is caused by a 50-residue deletion mutation at the C-terminus of lamin A (LA Δ 50). Nuclei in LA Δ 50 mutant cells display a loss of heterochromatin, indicating that the C-terminal region of lamin A/C plays a role in chromatin organization. ProAR-dependent heterochromatin formation might be mediated via regulation of lamin function. Heterochromatinization is nearly synonymous with epigenetic gene silencing; however, there are some reports that suggest heterochromatin formation is required for activation of gene expression (Weiler and Wakimoto, 1995; Lu et al., 2000; Yasuhara and Wakimoto, 2006).

AR has been characterized as a growth factor involved in pathophysiological cell proliferation and differentiation. Thus, proAR translocation into the INM and interaction with lamin A/C, resulting in the induction of heterochromatinization and global transcriptional suppression, might participate in dynamic epigenetic reprogramming of gene expression during cell proliferation and differentiation.

Materials and Methods

Plasmids

Human *AREG* cDNA was subcloned into the *EcoRI-XhoI* sites of the pME18s vector (full-length *AREG*) or V5-tagged-pME18s vector (AR-V5). AR-V5-C was generated by insertion of a sequence encoding the V5-tag using PCR with appropriate oligonucleotide DNA primers producing the coding sequence. Mutants of truncated forms of *AREG* were generated using PCR and inserted into the *EcoRI-XhoI* sites of pME18s (AR- Δ C-V5) or pME18S-V5 (AR- Δ C1-V5~AR- Δ C4-V5). The 10- or 11-residue deletion mutant of *AREG* or AR-V5-C were generated using PCR and inserted into the *EcoRI-XhoI* sites of pME18s (AR- Δ C11, AR-V5- Δ C10, and AR-V5- Δ C11). Mutants with Ala substitutions at Lys239 and Lys240 of wild-type *AREG* (AR-m2) were generated using PCR-based site directed mutagenesis. All the cDNA constructs were verified by DNA sequencing using a CEQ 8000 DNA Analysis System (Beckman Coulter). pGEX-lamin A derivatives were gifts from Toshinori Ozaki (Ozaki et al., 1994). The plasmids pME 18S-HB-EGF-V5-C and pEGF-C1-hLAP2 β were described previously (Hieda et al., 2008).

Antibodies

Affinity-purified rabbit pAbs against synthetic peptides corresponding to the cytoplasmic region of proAR (residues 233-250), i.e. AR-C pAb was obtained from Immuno Biological Laboratories (IBL). Goat pAb against the extracellular region of proAR, anti-AR-N pAb was from R&D Systems. The mouse anti-V5 mAb was from Invitrogen. Mouse anti-lamin A/C mAb (636), the mouse anti-LAMP-1 mAb (H4A3) and goat anti-lamin B pAb (M-20) were from Santa Cruz Biotechnology. Mouse anti- β -actin mAb (AC-15) was from Sigma. Sheep anti-human TGN46 pAb was from Serotec. Mouse anti-GM130 mAb was from BD Transduction Laboratories. HRP-conjugated secondary Abs were from Promega. FITC- or Cy3-conjugated secondary antibodies were from Jackson Immuno Research Laboratories. Anti-HP1 β rat mAb (MAC35), anti-histone H3 dimethyl K9 mAb (1220), anti-histone H3 trimethyl K9 pAb were from Abcam. Rabbit anti-GFP pAb was from Medical & Biological Laboratories (MBL).

Preparation of monoclonal antibodies

Monoclonal antibodies were generated using the rat lymph node method (Sado et al., 1995). Briefly, WKY/Ncrj rats (Charles River Japan) were immunized with an emulsion containing a KLH-conjugated synthetic peptide, which corresponds to that of the cytoplasmic region of AR, and Freund's complete adjuvant. Cells from the lymph nodes were fused with mouse myeloma Sp2/0 cells. The hybridomas were screened using ELISA and three clones (No.2, No.5 and No.9) were established. The isotype subclass of mAb2, mAb5 and mAb9 were determined to be IgM, IgG2b and IgG1, respectively, using a rat monoclonal antibody ID/SP Kit (Zymed Laboratories).

Cell culture, transfection and TPA treatment

HeLa cells were grown in DMEM (Nikken Biomedical Laboratory) supplemented with 10% fetal bovine serum (HyClone). Transfections were performed using LipofectamineTM 2000 (Invitrogen). For induction of the shedding process, cells were incubated with 100 nM TPA for 30 minutes, unless otherwise indicated in the figure legends.

Digitonin permeabilization

For digitonin permeabilization, the cells were incubated with transport buffer (20 mM HEPES pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM EDTA and 2 mM DTT) containing 33 μ g/ml digitonin on ice for 5 minutes. After permeabilization, cells were washed and fixed with 4% paraformaldehyde in transport buffer.

Preparation of cell lysate

Cells were lysed in ice-cold lysis buffer, PBS (-) containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, and protease inhibitor cocktail (Roche). Cell lysates were incubated for 30 minutes at 4°C and centrifuged at 9100 g for 10 minutes. The resulting supernatants were used as total cell lysates.

Immunoprecipitation

Total cell lysates were incubated with anti-lamin A/C mAb for 2 hours at 4°C. Protein-G-Sepharose beads (Amersham Biosciences) were then added to the mixture. After incubation for 1 hour, the suspension was centrifuged, and the collected protein-G-Sepharose beads were washed three times with lysis buffer. The bound proteins were analyzed by western blotting.

GST pull-down assays

GST and GST-lamin mutants were produced in and purified from *E. coli*, as described previously (Ozaki et al., 1994). Total cell lysates were mixed with 2 μ g recombinant GST or GST-lamin mutants and glutathione-Sepharose beads for 2 hours at 4°C. The bound proteins were analyzed by SDS-PAGE, followed by western blotting.

Immunofluorescence microscopy

Cells were fixed with 4% paraformaldehyde for 20 minutes and permeabilized with 0.2% Triton X-100 for 10 minutes at room temperature. Cells were blocked with 2% BSA and incubated with primary antibodies overnight at 4°C. Cells were viewed with an epifluorescence microscope (IX70, Olympus).

siRNA knockdown

A specific RNA dimer that targets the coding sequence of human A-type lamin at nucleotides 608-626 was obtained from Dharmacon. Specific RNA dimers corresponding to B-type lamins, lamin B1 and lamin B2 (Tsai et al., 2006) were obtained from B-Bridge International. RNA dimers were transfected into HeLa cells using Lipofectamine2000 (Invitrogen).

Br-U incorporation and its quantification

Cells were grown in 5 mM Br-U for 30 minutes and fixed with 4% paraformaldehyde. Br-RNA was immunolabeled using a rat anti-BrdU mAb (Abcam). The fluorescent intensity in the nucleus was measured in 200 cells with Image-pro plus (MediaCybernetics). The analysis was performed in triplicate and results were considered significant at a level of $P < 0.05$.

Nuclear extraction

HeLa cells transiently expressing proAR were extracted using a protocol described previously (Markiewicz et al., 2005) with some modifications. Cells were rinsed twice with TM buffer (20 mM Tris-HCl pH 7.5, 3 mM MgCl₂) and then incubated for 10 minutes on ice in TM buffer containing 0.4% Triton X-100, 0.5 mM CuCl₂ and proteinase inhibitors. After washing with TM buffer, cells were incubated with DNase I (20 U/ml) for 20 minutes at 37°C, or/and incubated with 2 M NaCl for 5 minutes on ice.

Immunogold electron microscopy

The cells were fixed with 2% paraformaldehyde/0.25% glutaraldehyde, dehydrated and embedded in LR White resin. Ultrathin sections were blocked with 1% BSA and incubated in anti-V5 mAb. The bound antibody was detected using gold-conjugated anti-mouse IgG (15 nm; BBIInternational, UK). After incubation, the grids were counterstained with 2% uranyl acetate and lead citrate and examined with a JEOL JEM1230 transmission electron microscope.

pGEX-lamin A derivatives were a gift from Toshinori Ozaki (Chiba Cancer Research Institute). Mouse myeloma cells (SP2/0) were a gift from Yoshikazu Sado (Shigei Medical Research Institute). We thank Hidehiko Iwabuki for technical help with plasmid construction and Higashiyama's laboratory members for useful discussion. This study was supported by Grants-in Aid for Scientific Research No.19570182 to MH, and No. 17014068 and No. 17390081 to S.H. from the Ministry of Education, Culture, Sports, Science and Technology and from Japan Society for the Promotion of Science, and Precursory Research for Embryonic Science and Technology (Information and Cell Function), JST, Japan.

References

- Adam, S. A., Marr, R. S. and Gerace, L. (1990). Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. *J. Cell Biol.* **111**, 807-816.
- Anklesaria, P., Teixidó, J., Laiho, M., Pierce, J. H., Greenberger, J. S. and Massagué, J. (1990). Cell-cell adhesion mediated by binding of membrane-anchored transforming growth factor alpha to epidermal growth factor receptors promotes cell proliferation. *Proc. Natl. Acad. Sci. USA* **87**, 3289-3293.
- Blobel, C. P. (2005). ADAMs: key components in EGFR signaling and development. *Nat. Rev. Mol. Cell Biol.* **6**, 32-43.
- Dreuillet, C., Tillit, J., Kress, M. and Ernout-Lange, M. (2002). *In vivo* and *in vitro* interaction between human transcription factor MOK2 and nuclear lamin A/C. *Nucleic Acids Res.* **30**, 4634-4642.
- Dyer, J. A., Kill, I. R., Pugh, G., Quinlan, R. A., Lane, E. B. and Hutchison, C. J. (1997). Cell cycle changes in A-type lamin associations detected in human dermal fibroblasts using monoclonal antibodies. *Chromosome Res.* **6**, 383-394.
- Goldman, R. D., Gruenbaum, Y., Moir, R. D., Shumaker, D. K. and Spann, T. P. (2002). Nuclear lamins: building blocks of nuclear architecture. *Genes. Dev.* **16**, 533-547.
- Gruenbaum, Y., Margalit, A., Goldman, R. D., Shumaker, D. K. and Wilson, K. L. (2005). The nuclear lamina comes of age. *Nat. Rev. Mol. Cell Biol.* **6**, 21-31.
- Hieda, M., Isokane, M., Koizumi, M., Higashi, C., Tachibana, T., Shudou, M., Taguchi, T., Hieda, Y. and Higashiyama, S. (2008). Membrane-anchored growth factor, HB-EGF, on the cell surface targeted to the inner nuclear membrane. *J. Cell Biol.* **180**, 763-769.
- Higashiyama, S., Iwamoto, R., Goishi, K., Raab, G., Taniguchi, N., Klagsbrun, M. and Mekada, E. (1995). The membrane protein CD9/DRAP27 potentiates the juxtacrine

- growth factor activity of the membrane-anchored heparin-binding EGF-like growth factor. *J. Cell Biol.* **128**, 929-938.
- Higashiyama, S., Iwabuki, H., Morimoto, C., Hieda, M., Inoue, H. and Matsushita, N.** (2008). Membrane-anchored growth factors, the EGF family: beyond receptor ligands. *Cancer Sci.* **99**, 214-220.
- Hinkle, C. L., Sunnarborg, S. W., Loiselle, D., Parker, C. E., Stevenson, M., Russell, W. E. and Lee, D. C.** (2004). Selective roles for tumor necrosis factor alpha-converting enzyme/ADAM17 in the shedding of the epidermal growth factor receptor ligand family: the juxtamembrane stalk determines cleavage efficiency. *J. Biol. Chem.* **279**, 24179-24188.
- Holmer, L. and Worman, H. J.** (2001). Inner nuclear membrane proteins: functions and targeting. *Cell Mol. Life Sci.* **58**, 1741-1747.
- Hozák, P., Sasseville, A. M., Raymond, Y. and Cook, P. R.** (1995). Lamin proteins form an internal nucleoskeleton as well as a peripheral lamina in human cells. *J. Cell Sci.* **108**, 635-644.
- Iborra, F. J., Jackson, D. A. and Cook, P. R.** (1998). The path of transcripts from extranuclear synthetic sites to nuclear pores: transcripts in transit are concentrated in discrete structures containing SR proteins. *J. Cell Sci.* **111**, 2269-2282.
- Jackson, M. R., Nilsson, T. and Peterson, P. A.** (1990). Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. *EMBO J.* **9**, 3153-3162.
- King, M. C., Lusk, C. P. and Blobel, G.** (2006). Karyopherin-mediated import of integral inner nuclear membrane proteins. *Nature* **442**, 1003-1007.
- Kinugasa, Y., Hieda, M., Hori, M. and Higashiyama, S.** (2007). The carboxyl-terminal fragment of pro-HB-EGF reverses Bcl6-mediated gene repression. *J. Biol. Chem.* **282**, 14797-14806.
- Koberna, K., Stanek, D., Malinský, J., Eltsov, M., Pliss, A., Ctrnáctá, V., Cermanová, S. and Raska, I.** (1999). Nuclear organization studied with the help of a hypotonic shift: its use permits hydrophilic molecules to enter into living cells. *Chromosoma* **108**, 325-333.
- Kumaran, R. I., Muralikrishna, B. and Parnaik, V. K.** (2002). Lamin A/C speckles mediate spatial organization of splicing factor compartments and RNA polymerase II transcription. *J. Cell Biol.* **159**, 783-793.
- Lu, B. Y., Emtage, P. C., Duyf, B. J., Hilliker, A. J. and Eisenberg, J. C.** (2000). Heterochromatin protein 1 is required for the normal expression of two heterochromatin genes in *Drosophila*. *Genetics* **155**, 699-708.
- Markiewicz, E., Ledran, M. and Hutchison C. J.** (2005). Remodelling of the nuclear lamina and nucleoskeleton is required for skeletal muscle differentiation in vitro. *J. Cell Sci.* **118**, 409-420.
- Mounkes, L., Kozlov, S., Burke, B. and Stewart, C. L.** (2003). The laminopathies: nuclear structure meets disease. *Curr. Opin. Genet. Dev.* **13**, 223-230.
- Nanba, D., Mammoto, A., Hashimoto, K. and Higashiyama, S.** (2003). Proteolytic release of the carboxy-terminal fragment of proHB-EGF causes nuclear export of PLZF. *J. Cell Biol.* **163**, 489-502.
- Nilsson, T., Jackson, M. and Peterson, P. A.** (1989). Short cytoplasmic sequences serve as retention signals for transmembrane proteins in the endoplasmic reticulum. *Cell* **58**, 707-718.
- Ozaki, T., Saijo, M., Murakami, K., Enomoto, H., Taya, Y. and Sakiyama, S.** (1994). Complex formation between lamin A and the retinoblastoma gene product: identification of the domain on lamin A required for its interaction. *Oncogene* **9**, 2649-2653.
- Plowman, G. D., Green, J. M., McDonald, V. L., Neubauer, M. G., Disteche, C. M., Todaro, G. J. and Shoyab, M.** (1990). The amphiregulin gene encodes a novel epidermal growth factor-related protein with tumor-inhibitory activity. *Mol. Cell. Biol.* **10**, 1969-1981.
- Rozenfurt, E.** (2007). Mitogenic signaling pathways induced by G protein-coupled receptors. *J. Cell Physiol.* **213**, 589-602.
- Sado, Y., Kagawa, M., Kishiro, Y., Sugihara, K., Naito, I., Seyer, J. M., Sugimoto, M., Oohashi, T. and Ninomiya, Y.** (1995). Establishment by the rat lymph node method of epitope-defined monoclonal antibodies recognizing the six different alpha chains of human type IV collagen. *Histochem. Cell Biol.* **104**, 267-275.
- Sahin, U., Weskamp, G., Kelly, K., Zhou, H. M., Higashiyama, S., Peschon, J., Hartmann, D., Saftig, P. and Blobel, C. P.** (2004). Distinct roles for ADAM10 and ADAM17 in ectodomain shedding of six EGFR ligands. *J. Cell Biol.* **164**, 69-79.
- Sandvig, K. and van Deurs, B.** (2002). Transport of protein toxins into cells: pathways used by ricin, cholera toxin and Shiga toxin. *FEBS Lett.* **529**, 49-53.
- Schirmer, E. C., Florens, L., Guan, T., Yates, J. R. and Gerace, L.** (2003). Nuclear membrane proteins with potential disease links found by subtractive proteomics. *Science* **301**, 1380-1382.
- Singh, A. B. and Harris, R. C.** (2005). Autocrine, paracrine and juxtacrine signaling by EGFR ligands. *Cell Signal* **17**, 1183-1193.
- Spann, T. P., Moir, R. D., Goldman, A. E., Stick, R. and Goldman, R. D.** (1997). Disruption of nuclear lamin organization alters the distribution of replication factors and inhibits DNA synthesis. *J. Cell Biol.* **136**, 1201-1212.
- Spann, T. P., Goldman, A. E., Wang, C., Huang, S. and Goldman, R. D.** (2002). Alteration of nuclear lamin organization inhibits RNA polymerase II-dependent transcription. *J. Cell Biol.* **156**, 603-608.
- Tokumaru, S., Higashiyama, S., Endo, T., Nakagawa, T., Miyagawa, J. I., Yamamori, K., Hanakawa, Y., Ohmoto, H., Yoshino, K., Shirakata, Y. et al.** (2000). Ectodomain shedding of epidermal growth factor receptor ligands is required for keratinocyte migration in cutaneous wound healing. *J. Cell Biol.* **151**, 209-220.
- Tsai, M. Y., Wang, S., Heidinger, J. M., Shumaker, D. K., Adam, S. A., Goldman, R. D. and Zheng, Y.** (2006). A mitotic Lamin B matrix induced by RanGTP required for spindle assembly. *Science* **311**, 1887-1893.
- Vlcek, S., Dechat, T. and Foisner, R.** (2001). Nuclear envelope and nuclear matrix: interactions and dynamics. *Cell Mol. Life Sci.* **58**, 1758-1765.
- Weiler, K. S. and Wakimoto, B. T.** (1995). Heterochromatin and gene expression in *Drosophila*. *Annu. Rev. Genet.* **29**, 577-605.
- Yasuhara, J. C. and Wakimoto, B. T.** (2006). Oxymoron no more: the expanding world of heterochromatic genes. *Trends Genet.* **22**, 330-338.
- Zastrow, M. S., Vlcek, S. and Wilson, K. L.** (2004). Proteins that bind A-type lamins: integrating isolated clues. *J. Cell Sci.* **117**, 979-987.