**Plexin-B1 signalling promotes androgen receptor translocation to the nucleus.**

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**Abstract**

Semaphorins and their receptors, plexins have diverse roles in many cancers affecting tumour growth, metastasis and angiogenesis. Plexin-B1, the receptor for semaphorin4D (Sema4D), has been implicated in prostate cancer where mutation of the gene and overexpression of the protein occur. It is not clear however which of the several Sema4D-activated signalling pathways downstream of plexin-B1 function in prostate cancer progression. We show here that Sema4D/plexin-B1 increases the expression of androgen-responsive genes and activates the transcriptional activity of the androgen receptor. Activation of plexin-B1 results in phosphorylation of the androgen receptor at Serine 81, a site which is phosphorylated by nuclear kinases. Cell fractionation and immunocytochemistry studies demonstrated that the proportion of cells with AR in the nucleus increases significantly upon Sema4D treatment. The N-terminal (AF-1) domain of AR, which contains binding sites for transcription regulators, is not required for this response. Depletion of AR suppressed Sema4D-induced anchorage-independent growth of LNCaP and LNCaP-LN3 cells, demonstrating the functional significance of these findings. These results show that Sema4D/plexin-B1 signalling promotes the translocation of the androgen receptor to the nucleus and thereby enhances AR transcriptional activity. Plexin-B1 is therefore a promising target for cancer therapy especially in low androgen situations such as those imposed by androgen deprivation therapy.

**Introduction**

Prostate cancer is curable when localised to the prostate but incurable once it has metastasised, highlighting the need for therapies inhibiting the spread of prostate cancer. Androgen receptor (AR) signalling is critical to the progression of prostate cancer and patients with metastatic prostate cancer respond initially to androgen deprivation therapy (ADT). However, castrate-resistant prostate cancer (CRPC) generally resumes after 2-3yrs of ADT due to androgen independent reactivation of the AR pathway.

Plexins are transmembrane receptors for semaphorins1, which act as chemotactic cues for cell movement. Plexin-B1, a receptor for semaphorin4D (Sema4D), activates the oncogenes ErbB22 and c-Met3 and regulates the small GTPases Rac4, R-Ras5, Rnd6, RhoA7 and Rap18, resulting in changes in the actin cytoskeleton and cell motility.

Plexin-B1 has diverse roles in cancer9: Plexin-B1 promotes tumour progression in ErbB2 positive breast cancer10 and ovarian cancer11 where progression and poor prognosis is associated with overexpression of the protein. Sema4D/Plexin-B1 signalling via ErbB2 promotes the invasive phenotype of breast cancer cells by activating RhoA or RhoC and knockdown of plexin-B1 expressionin mouse models of ErbB2-overexpressing breast cancer reduces metastasis10. In contrast, plexin-B1 acts as a tumour suppressor gene in estrogen receptor-positive breast cancer12, and in melanoma13 and progression of melanoma is associated with a decrease in plexin-B1 expression and plexin-B1 signalling inhibits the oncogenic HGF- c-met pathway14. The mechanism by which plexin-B1 contributes to cancer progression in each cancer type is therefore dependent on cellular context and on which plexin-B1 signalling pathways predominate in the tumour cell.

Plexin-B1 has also been implicated in prostate cancer: somatic missense mutations in the plexin-B1 gene and overexpression of the protein occur in prostate tumours15. It is not yet clear, however, which of the many Sema4D/Plexin-B1 activated pathways downstream of plexin-B1 contribute to prostate cancer progression specifically and at which stage(s) of prostate cancer they operate.

The mutations in plexin-B1 found in prostate cancer result in the loss of plexin-B1-mediated pathways that inhibit cell migration (including those involving Rac4, Rnd6, R-Ras5) but do not affect promigratory pathways such as those involving ErbB2 and Rho16.Plexin-B1 interacts with the ErbB2 in the prostate cancer cell line, LNCaP, and Sema4D induces the phosphorylation of ErbB2 in this cell line. Furthermore, knockdown of ErbB2 expression inhibits the Sema4D-induced motility of LNCaP cells, suggesting that activation of the Sema4D/plexin-B1 signalling pathway involving ErbB2 promotes the invasive behaviour of prostate cancer cells17. ErbB2 overexpression occurs in late stage prostate cancer as do mutations in plexin-B1. Sema4D/plexin-B1 signalling may therefore increase the invasive characteristics of prostate cancer cells via activation of ErbB2 and Rho and via mutation of plexin-B1 in late stage prostate cancer, in a similar way to breast cancer.

However, there is also evidence for an additional role for plexin-B1 in the earlier stages of prostate cancer. Increased levels of plexin-B1 protein appear at an earlier stage than the low level ErbB2 overexpression in prostate tumours15 and, unlike breast cancer, the ErbB2 gene is not amplified18. Knockdown of plexin-B1 in LNCaP cells inhibits proliferation under anchorage independent conditions17, a phenotype not associated with ErbB2 activation. Higher levels of wild-type plexin-B1 expression may therefore contribute to prostate cancer progression at the earlier stages via pathways other than those involving ErbB2. We sought to investigate whether other prostate cancer-specific pathways were activated in prostate cancer cells by plexin-B1.

**Results and Discussion**

**Sema4D affects expression of androgen-responsive genes**

In preliminary experiments to discover which signalling pathways are activated by plexin-B1 in prostate cancer cells, we screened a panel of genes known to be involved in prostate cancer for changes in expression in response to Sema4D treatment. Expression of the prostate cancer-associated genes was compared in LNCaP-LN3 cells expressing shRNA to plexin-B1 with LNCaP-LN3 cells expressing non-silencing control shRNA and treated with Sema4D, using a RT-qPCR array. A change in expression of several genes that are known to be regulated by AR signalling was observed, including upregulation of KLK319, AR20, B2M21, FASN22, acetyl CoA carboxylase  (ACACA)23 and EGFR24 and a marked downregulation of IGFBP525, 26 (data not shown).

To confirm these initial findings, mRNA expression of two of these androgen-responsive genes was assessed in LNCaP and LNCap-LN3 cells in response to plexin-B1 activation by Sema4D, by RT-qPCR. LNCaP-LN3, a derivative of the prostate cancer cell line LNCaP, has lost the mutant allele of plexin-B1 found in LNCaP and expresses wild-type (WT) plexin-B1 only17.

LNCaP and LNCap-LN3 cells were serum starved and then treated with Sema4D (2g/ml), for 48hrs or with PBS or dihydrotestosterone (DHT, 1nM) as controls. Expression of KLK3 increased significantly in both cell lines following treatment with Sema4D (fig1A). LNCaP and LNCaP-LN3 cells produce endogenous Sema4D and activate plexin-B1 via an autocrine/paracrine mechanism27. Knockdown of plexin-B1 by shRNA in both cell lines decreased KLK3 expression, confirming that the Sema4D-induced effect on KLK3 expression and possibly AR activation is dependent on plexin-B1 expression (fig1A).

IGFBP5 expression is decreased by androgen receptor activation25, 26. A decrease in IGFBP5 expression was observed in both LNCaP and LNCaP-LN3 cells upon Sema4D treatment (fig1B), as assessed by RT-qPCR. Consistent with these findings, knockdown of plexin-B1 by shRNA in both cell lines increased IGFBP5 expression.

**ErbB2 is not required for Sema4D/plexin-B1-mediated activation of the androgen receptor**

One mechanism by which the AR pathway is upregulated in CRPC is through activation of AR by ErbB228. ErbB2 interacts with and is activated by plexin-B1 in LNCaP and LNCaP-LN3 prostate cancer cells as it is in other cell types29. To determine if Sema4D activation of AR is mediated by ErbB2 the effect of ErbB2 knockdown on Sema4D-induced AR activity was monitored. The level of KLK3 mRNA was measured in LNCaP and LNCap-LN3 cells in which ErbB2 expression was knocked down by two different shRNAs. Knockdown of ErbB2 using either shRNAs had little effect on expression of KLK3 in LNCaP or LNCaP-LN3 (fig1C). ErbB2 expression cannot therefore account for all the increase in AR activation in these cells.

**Sema4D increases androgen receptor transcriptional activity**

The KLK3 and IGFBP5 genes are activated and repressed respectively by AR. To determine if Sema4D/plexinB signalling affects AR transcriptional activity, an inducible reporter construct that encodes a luciferase-reporter gene downstream of a promoter containing androgen response elements (AREs) was used. LNCaP and LNCaP-LN3 cells were transduced with lentivirus containing an ARE-luciferase or control-luciferase construct lacking the ARE domain, together with a renilla expression vector to control for transduction efficiency. Luciferase activity was monitored in response to Sema4D stimulation. A significant increase in luminescence in Sema4D-treated cells was observed relative to unstimulated cells (fig2A) for both cell lines. These results indicate that Sema4D/plexin-B1 signalling enhances transcription from ARE-containing promoters.

**Sema4D/plexin-B1 signals via AR to promote anchorage independent growth.**

Stimulation with Sema4D increases the proliferation of LNCaP and LNCaP-LN3 cells under anchorage independent conditions27. To determine if AR is involved in this process, AR expression was knocked down by shRNA and growth of the cells in agarose measured in the presence and absence of Sema4D. A significant increase in colony growth was observed with Sema4D in cells infected with lentivirus expressing control nonsilencing shRNA, as expected. Colonies in which AR expression had been knocked down by shRNA were smaller. Sema4D did not increase anchorage-independent growth of these cells (fig2B). These results show that Sema4D-induced proliferation of LNCaP and LNCap-LN3 cells under anchorage independent conditions is dependent on AR and suggests that plexin-B1 signals via AR to promote this phenotype.

**Sema4D increases the phosphorylation of AR**

AR is phosphorylated at Serine-81 leading to activation of AR30. In order to define the mechanism by which Sema4D activates AR the effect of Sema4D on phosphorylation of AR was examined. Treatment of LNCaP and LNCaP-LN3 with Sema4D resulted in an increase in phosphorylation of AR(Ser81) as assessed by immunoblotting (fig3A). AR(Ser81) phosphorylation did not increase with Sema4D treatment in cells in which expression of plexin-B1 had been knocked down by shRNA (fig3B), indicating that Sema4D-induced AR(Ser81) phosphorylation is dependent on plexin-B1.

**Sema4D increases translocation of AR to the nucleus**

AR is a nuclear receptor that, upon ligand binding in the cytoplasm, moves to the nucleus of the cell where it acts as a transcription factor. Since AR is phosphorylated at Ser81 by CDK1 and CDK931, 32, both kinases that are located in the nucleus, we next investigated whether Sema4D/plexin-B1 signalling affects nuclear translocation of AR. LNCaP and LNCaP-LN3 were treated with Sema4D (2g/ml) or PBS or DHT (1nM) and then the extracted protein preparation was separated into cytoplasmic, membrane, nuclear and chromatin-bound fractions. An increase in both phosphorylated and non-phosphorylated forms of AR was found in the nucleus upon Sema4D treatment (fig4A).

To further investigate the increase in nuclear AR upon Sema4D treatment, we transfected the prostate cancer cell line PC3 (which does not express endogenous AR) with an AR expression vector expressing full length AR and treated the transfected cells with PBS, Sema4D (2g/ml) or DHT (1nM). The subcellular localisation of the AR protein in transfected cells was assessed by immunocytochemistry using confocal microscopy. Cells were scored blind and categorised into three groups: 1) nuclear staining > cytoplasmic staining, 2) nuclear and cytoplasmic staining equal, 3) cytoplasmic staining > nuclear staining. A significant increase in nuclear staining was observed following treatment with Sema4D (fig4B).

In initial steps to identify which domains of the AR are required for Sema4D-induced translocation of AR to the nucleus, the experiment was repeated with a DKK-tagged AR construct encoding isoform 2 of AR, a splice variant which lacks the N-terminal domain (AF-1). Using this smaller construct, nuclear translocation of AR was seen in a higher proportion of cells following both Sema4D and DHT treatment. A significant increase in nuclear staining was observed in transfected cells treated with Sema4D (fig4C, 4D) compared with control.

Overall, our results show that, upon Sema4D stimulation, plexin-B1 promotes the translocation of AR to the nucleus, in the absence of added androgen, resulting in an increase in phosphorylation of AR(Ser81) and an increase in transcription of androgen-responsive genes. The N-terminal (AF-1) domain of AR, which contains binding sites for transcription regulators, is not required for this response. Furthermore, Sema4D/plexin-B1 signals via AR to promote anchorage–independent growth.

These results suggest that plexins may function in longer term signalling by regulating gene expression in addition to their well-documented role in short term signalling controlling the actin cytoskeleton, growth cone collapse and cell motility via RhoGTPases.

It is not known how Sema4D/plexin-B1 influences AR nuclear trafficking. The AR moves towards the nucleus upon ligand binding by tracking along microtubules using the minus-end directed motor protein, dynein. Entry and exit of AR through the nuclear pore complex is mediated by binding of importins and exportins, a process that is regulated by the small GTPase Ran. Plexins have been implicated in both microtubule and RanGTPase systems. Plexins affect microtubule dynamics in a number of ways: i) by binding to the EB family of microtubule + end binding proteins33, ii) by activating GSK334 and iii) by activating CRMP proteins which interact with tubulin and can regulate microtubule assembly35. Plexins are also implicated in the RanGTPase system: plexins of the ‘A’ class, as well as AR, interact with the RanGTPase binding protein RanBPM which is involved in nuclear trafficking by binding to RanGTP and by affecting dynein activity36. Plexins also have a functional role in retro and anterograde axonal trafficking37.

Androgen deprivation therapy is the standard therapy for progressive prostate cancer, highlighting the importance of the AR signalling pathway in prostate tumours. The eventual resistance to this therapy arises from ligand independent AR signalling. To function, the AR must enter the nucleus where it acts as a transcription factor and activates gene transcription. Factors that influence translocation of AR to the nucleus are consequently potential therapeutic targets. Taxanes, such as docetaxel and prednisone, chemotherapy drugs which improve survival of patients with CRPC, for example, bind to tubulin and suppress microtubule dependent trafficking of AR to the nucleus38. Plexin-B1 may contribute to prostate cancer progression by enhancing AR translocation to the nucleus especially under low androgen conditions and thus may be important in resistance to androgen deprivation therapy.

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**Figures**

**Figure 1. Sema4D modulates expression of androgen-responsive genes**

**A. Sema4D modulates the expression of the androgen-responsive gene KLK3.** i) Sema4D increases the expression of KLK3 in LNCaP and LNCap-LN3 cells. Serum starved cells17 were treated with PBS, Sema4D (R&D, 2g/ml) or dihydrotestosterone (DHT, Sigma, 1nM) for 48 hours. KLK3 mRNA levels were measured by quantitative RT-QPCR**.** RNA (500ng) was reverse transcribed (Superscript III, Invitrogen) and RT-qPCR performed using Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent) on an Eppendorf Mastercycler ep Realplex machine. Copy numbers were derived from standard curves. Primers: *KLK3 Fwd:- AACCAGAGGAGTTCTTGACC, Rev:- AGCACACAGCATGAACTTG.* Each data point was normalised to the geometric mean of 4 reference genes, amplified in parallel (qStandard): GAPDH, RPL32, SDHA, TBP, identified as stable using geNorm. Primers: GAPDH: *TGCACCACCAACTGCTTAGC; GGCATGGACTGTGGTCATGAG*. RPL32: *CATCTCCTTCTCGGCATCAT; ACCCTGTTGTCAATGCCTCT.* SDHA: AGAAGCCCTTTGAGGAGCA; CGATCACGGGTCTATATTCCAGA. TBP: GAACATCATGGATCAGAACAACAG; ATAGGGATTCCGGGAGTCAT. B2M: CTCTCTCTTTCTGGCCTGGAG; ACCCAGACACATAGCAATTCAG. Average of three experiments in duplicate; \*p<0.05 (Ttest) vs PBS control.ii) Knockdown of plexin-B1 expression by two different shRNAs, detected by immunoblotting using plexin-B1 (ECM Biosciences) and anti-β-actin (Abcam, ab6276) antibodies. shRNA was produced in 293FT cells using lentiviral-shRNA target set RHS4533-NM-001130082 (GE Healthcare) or a non-silencing control shRNA, with a trans-lentiviral GIPZ packaging system (Thermo). Cells were selected with puromycin (10g/ml, Sigma). iii) Knockdown of plexin-B1 expression by two different shRNAs decreases expression of KLK3. Expression was measured by quantitative RT-QPCR (as in i). Average of three experiments in duplicate; \*p<0.05 (Ttest) vs non-silencing control. **B. Sema4D modulates the expression of the androgen-responsive gene IGFBP5.** i) Sema4D decreases the expression of IGFBP5 in LNCaP and LNCap-LN3 cells. Serum starved cells were treated with PBS or Sema4D (2g/ml) or DHT (1nM) for 48 hours and mRNA levels measured by quantitative RT-QPCR (as in A), primers: *IGFBP5 Fwd:- CCGCGAGCAAGTCAAGATCG,* *Rev:*- *TCAGCTCGGAGATGCGGGTG.* Average of four experiments in duplicate; \*p<0.05 (Ttest) vs PBS control.ii) Knockdown of plexin-B1 by shRNA increases expression levels of IGFBP5 in LNCaP and LNCap-LN3 cells, measured by quantitative RT-QPCR (as in A). Average of three experiments in duplicate; \*p<0.05 (Ttest) vs non-silencing control. **C. ErbB2 is not required for Sema4D/plexin-B1-mediated activation of the androgen receptor**  i) Knockdown of ErbB2 by shRNA has little effect on the expression levels of KLK3 . ErbB2 expression was knocked down with Lentiviral-shRNA target set RHS4533- [NM\_001005862](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide&cmd=search&term=NM_001005862) (GE Healthcare)) and KLK3 expression monitored by RT-qPCR (as in A), average of three experiments. ii) Knockdown of ErbB2 by two different shRNAs, detected by immunoblotting using ErbB2 (Millipore), anti-β-actin (Abcam, ab6276) antibodies.

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**Figure 2. A. Sema4D treatment increases the transcription of an ARE-luciferase reporter gene.**

AR activation of an ARE-luciferase reporter construct was assessed following treatment of transfected cells with PBS, Sema4D or DHT. 2x105 cells (LNCaP or LNCaP-LN3) were transduced with 8x105 TU of ARE-Luc and 6.4x104 TU renilla lentiviral particles, or 8x105 TU of negative control-Luc and 6.4x104 TU renilla lentiviral particles (Cignal lenti reporter assay Qiagen) and infected cells were selected with puromycin dihydrochloride (10g/ml, Sigma). For each assay, 4x104 (LNCaP) or 2x104 (LNCaP-LN3) cells per well of a 96 well plate expressing ARE-Luc and control-renilla or control-Luc and control-renilla wereserum starved for 2 nights, then treated with PBS, Sema4D (2g/ml) or DHT (1nM) for 2 hours. Luciferase activity was detected with Dual Glo luciferase detection reagent (Promega). Average of five experiments in triplicate; \*p<0.05 (Ttest) vs PBS control. **B.Sema4D/plexin-B1 signals via AR to promote anchorage independent growth.** LNCaP (i) or LNCap-LN3 (ii) cells expressing a non silencing shRNA control or two different shRNAs to AR were grown under anchorage-independent conditions (0.34% agarose) in the presence of Sema4D or control conditioned medium17. The lentiviral-shRNA target set RHS4531-EG367 (V2LHS\_239349 , V3LHS\_367662 (GE Healthcare)) was used to knock down AR expression. Knockdown was assessed by immunoblotting using AR (Millipore) and actin (Abcam) antibodies. The colonies were sized and quantified using a microscope and the mean size of colony for each cell line calculated. Colonies above the mean size were counted and the fold increase in numbers of colonies above the mean size with Sema4D vs no Sema4D calculated. Average of three experiments in duplicate; \*p<0.05 (Ttest) vs non-silencing control.

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**Figure 3. Sema4D treatment promotes phosphorylation of androgen receptor at Serine 81**

A). LNCaP and LNCaP-LN3 cells were treated with PBS or Sema4D (2ug/ml) or DHT (1nM) and the phosphorylation state of AR at Ser81 was assessed by immunoblotting using AR (Millipore), phospho-Ser81 (Millipore),anti-β-actin (Abcam, ab6276) antibodies.

B). Sema4D-induced AR(Ser81) phosphorylation is dependent on plexin-B1 signalling. Expression of plexin-B1 was knocked down by shRNA in LNCaP (i) and LNCaP-LN3 (ii) cells (fig1A), and the effect of Sema4D treatment on phosphorylation of AR(Ser81) monitored by immunoblotting.

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Description automatically generatedFigure 4. Sema4D increases the translocation of AR to the nucleus**.

**A).** Cells were treated with PBS or Sema4D(2g/ml) or DHT (1nM) and protein extracted from cytoplasmic and nuclear fractions (subcellular protein fractionation kit, Thermo Scientific). The subcellular localisation of AR and phospho-AR and AR(Ser81) was analysed by immunoblotting using AR (Millipore), phospho-Ser81 (Millipore), tubulin(Sigma), lamin (Sigma) antibodies. **B).** PC3 cells were transfected with AR(GFP) transcript variant 1 (Origene) and serum-starved cells were treated with PBS, Sema4D(2g/ml) or DHT(1nM) for 90mins. The cells were fixed (4% paraformaldehyde), permeabilised (0.2% triton), stained by immunofluorescence for AR-GFP with an anti-AR antibody (Millipore), then anti-rabbit-FITC secondary antibody (Southern Biotech), and with phalloidin-TRITC (Sigma) and DAPI. Cells were scored ‘blind’ to their treatment. Transfected cells were scored according to the following criteria: a). intensity of cytoplasmic staining exceeded that of nuclear staining (C>N), b). intensity of cytoplasmic staining was equal to that of nuclear staining (N=C), c). intensity of nuclear staining exceeded that of cytoplasmic staining (N>C). Slides were scored on a [Perkin Elmer Spinning Disc confocal microscope using Volocity Acquisition](http://www.ucl.ac.uk/wibr/services/confocal/perkin-elmer/index.htm) at 63x magnification. i). % cells where intensity of nuclear staining exceeded that of cytoplasmic staining (N>C), average of three experiments, 204 or more cells scored per experiment; \*p<0.01 (Ttest) vs PBS control. ii). relative proportion of cells where nuclear staining exceeded cytoplasmic staining (N>C), was equal to that of cytoplasmic staining (N=C), or was less than that of cytoplasmic staining (C>N). iii). Represented images of transfected cells showing cytoplasmic greater than nuclear staining (C>N, top), and of nuclear greater than cytoplasmic staining (N>C, lower image). **C).** PC3 cells transfected with AR transcript variant 2 (DDK-tagged), (Origene) were treated with PBS, Sema4D(2g/ml) or DHT(1nM) for 90mins and stained with anti-DKK antibody (Origene, FITC), phalloidin-TRITC and DAPI and scored as in B. i). % cells where intensity of nuclear staining exceeded that of cytoplasmic staining (N>C), average of three experiments, 86 or more cells scored per experiment; \*p<0.01 (Ttest) vs PBS control. ii). relative proportion of cells where nuclear exceeded cytoplasmic staining (N>C), was equal to that of nuclear staining (N=C), or was less than that of cytoplasmic staining (C>N). **D).** Representative micrographs of PC3 cells transfected with AR transcript variant 2 (DDK-tagged) and treated with PBS, Sema4D (2g/ml) or DHT(1nM), fixed and stained with anti-DKK antibody (FITC), phalloidin (TRITC) and DAPI.Scale bar = 11m

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