

Netrin-1 blockade inhibits tumor associated Myeloid-derived suppressor cells, cancer stemness and alleviates resistance to chemotherapy and immune checkpoint inhibitor

Benjamin Ducarouge, Anna-Rita Redavid, Camille Victoor, Ruxanda Chira, Aurélien Fonseca, Maëva Hervieu, Roméo Bergé, Justine Lengrand, Pauline Vieugué, David Neves, et al.

▶ To cite this version:

Benjamin Ducarouge, Anna-Rita Redavid, Camille Victoor, Ruxanda Chira, Aurélien Fonseca, et al.. Netrin-1 blockade inhibits tumor associated Myeloid-derived suppressor cells, cancer stemness and alleviates resistance to chemotherapy and immune checkpoint inhibitor. Cell Death and Differentiation, 2023, 30 (10), pp.2201-2212. 10.1038/s41418-023-01209-x . hal-04253805

HAL Id: hal-04253805 https://cnrs.hal.science/hal-04253805

Submitted on 23 Oct 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Netrin-1 blockade inhibits tumor associated Myeloid derived suppressor cells, cancer stemness and alleviates resistance to chemotherapy and immune checkpoint inhibitor

Benjamin Ducarouge^{1,2*}, Anna-Rita Redavid^{1*}, Camille Victoor^{1,2}, Ruxanda Chira^{1,2} 5 Aurélien Fonseca³, Maëva Hervieu¹, Roméo Bergé^{1,2}, Justine Lengrand^{1,2}, Pauline 6 Vieugué¹, David Neves², Isabelle Goddard¹, Mathieu Richaud¹, Pierre-Alexandre 7 Laval¹, Nicolas Rama¹, David Goldschneider², Andrea Paradisi¹, Nicolas Gourdin⁴, 8 Sylvie Chabaud⁵, Isabelle Treilleux⁵, Nicolas Gadot⁶, Isabelle Ray-Coquard⁶, Stéphane 9 Didier Decaudin⁷, Fariba Némati⁷, Elisabetta Marangoni⁷, Eliane Mery-10 Depil². Lamarche⁸, Catherine Génestie⁹, Séverine Tabone-Eglinger⁶, Mojgan Devouassoux-11 Shisheboran¹⁰, Kathryn J Moore¹¹, Benjamin Gibert^{1\$£}, Patrick Mehlen^{1,2\$£} and Agnes 12

13 Bernet^{1,2£}

14

¹Apoptosis, Cancer and Development Laboratory- Equipe labellisée 'La Ligue', Labex 15 16 DEVweCAN, Institut Convergence PLAsCAN, Centre de Cancérologie de Lyon, INSERM U1052-CNRS UMR5286, Université de Lyon, Centre Léon Bérard, 69008 17 Lyon, France. ²Netris Pharma, Centre Léon Bérard, 69008 Lyon, France. ³Research 18 Pathology Department, Centre Léon Bérard, Lyon, France. ⁴Targeting of the tumor and 19 its immune environnement, Centre de Cancérologie de Lyon, INSERM U1052-CNRS 20 21 UMR5286, Université de Lyon, Centre Léon Bérard, 69008 Lyon, France. ⁵UBET, Centre Léon Bérard, Lyon, France. ⁶Pathology Department, Centre Léon Bérard, Lyon, 22 France. ⁷Laboratory of Preclinical Investigations, Translational Research Department, 23 24 Institut Curie, Université Paris-Sciences-et-Lettres, 75005 Paris, France.⁸Department 25 of Pathology, IUCT Oncopole, Toulouse, France.⁹Department of Pathology, Gustave Roussy, Villejuif, France.¹⁰Hospices Civils de Lyon, Department of Pathology, 69002 26 Lyon, France ¹¹Department of Medicine, Leon H. Charney Division of Cardiology, New 27 York University School of Medicine, New York, New York, USA. 28 ^{\$}Corresponding authors: P. Mehlen; e-mail: patrick.mehlen@lyon.unicancer.fr , A. 29

30 Bernet; e-mail: <u>agnes.bernet@lyon.unicancer.fr</u> and B. Gibert; e-mail: 31 benjamin.gibert@lyon.unicancer.fr

- 32 [£]Co-senior authors
- 33

34 **Abstract**:

35

36

Drug resistance and cancer relapse represent significant therapeutic 37 38 challenges after chemotherapy or immunotherapy, and a major limiting factor 39 for long-term cancer survival. Netrin-1 was initially identified as a neuronal navigation cue but has more recently emerged as an interesting target for cancer 40 41 therapy, which is currently clinically investigated. We show here that netrin-1 is 42 an independent prognostic marker for clinical progression of breast and ovary 43 cancers. Cancer stem cells (CSCs)/Tumor initiating cells (TICs) are hypothesized 44 to be involved in clinical progression, tumor relapse and resistance. We found a significant correlation between netrin-1 expression and cancer stem cell (CSC) 45 markers levels. We also show in different mice models of resistance to 46 chemotherapies that netrin-1 interference using a therapeutic netrin-1 blocking 47 48 antibody alleviate resistance to chemotherapy and triggers an efficient delay in 49 tumor relapse and that this effect is associated with CSCs loss. We also 50 demonstrate that netrin-1 interference limits tumor resistance to immune 51 checkpoint inhibitor and provide evidence linking this enhanced anti-tumor 52 efficacy to the blockage of recruitment of a subtype of myeloid-derived suppressor cells (MDSCs) called PMN-MDSCs. We have functionally 53 54 demonstrated that these immune cells promote CSCs and, consequently, resistance to anti-cancer treatments. Together, these data support the view of 55 56 both a direct and indirect contribution of netrin-1 to cancer stemness and we propose that this may lead to therapeutic opportunities by combining 57 conventional chemotherapies and immunotherapies with netrin-1 interfering 58 59 druas.

61 **Introduction**:

62

Netrin-1 is a multifunctional, secreted, laminin-related glycoprotein that plays 63 key roles in neuronal navigation, angiogenesis, and cell survival¹⁻³. Netrin-1 is also 64 implicated in numerous pathologies including type 2 diabetes, cardiovascular disease, 65 and cancer⁴⁻⁹. Its activity has been shown to principally occur through the regulation of 66 67 the signaling pathways transduced by its main receptors, Deleted in Colorectal Carcinoma (DCC) and UNC5-Homolog (UNC5H- i.e., UNC5A, UNC5B, UNC5C, 68 UNC5D)^{10,11}. Notably, netrin-1 has been shown to be up-regulated in many tumor 69 70 types, and this up-regulation has been proposed to act as a selective mechanism that 71 blocks apoptosis induced by the dependence receptors DCC and UNC5B^{4,12}.

72 Efforts to develop drugs that inhibit the interaction of netrin-1 with its receptors have therefore been initiated. Several pre-clinical proof-of-concept studies have 73 74 highlighted that candidate drugs interfering with netrin-1-receptor interactions markedly inhibit tumor growth and metastasis^{13–16}. NP137, a netrin-1 targeted 75 76 monoclonal antibody strongly accumulate within tumors and was recently evaluated in 77 a phase I clinical trial among patients with advanced solid cancers^{17,18}. Interim results 78 included both excellent safety profiles and encouraging signs of clinical activity, 79 particularly for gynecological indications¹⁹. Benefits were seen in patients despite 80 highly advanced disease resistant to standard of care, including chemotherapies and 81 immune checkpoint inhibitors^{20,21}.

82 A common view is that resistance to these anti-proliferative drugs is intrinsically 83 linked to the presence of Cancer Stem Cells (CSCs) or Tumor-Initiating Cells (TICs) 84 within the tumors. The notion is that CSCs and TICs rely on the existence of a distinct 85 subset of tumor cells that possesses the capacity to sustain tumor growth²². 86 CSCs/TICs are thought to express specific markers at the cell surface and are 87 identified functionally based on their ability to propagate tumors when serially 88 transplanted into recipient mice²³. Whether CSCs are a distinct subset of cancer cells, or whether they represent a functional state of some cancer cells, remains a matter of 89 90 debate. To date, the general consensus is that CSCs are key to clinical progression 91 due to their ability to self-renew and resist chemotherapies or more recently to immune checkpoint inhibitors^{24–28}, thereby facilitating tumor relapse²⁹. 92

93 In the present study, we thus investigated netrin-1 implication in breast and 94 ovary and its putative link with cancer stemness and resistance to

95 chemotherapy/immunotherapy. We show that netrin-1 expressed by cancer stem cells 96 promotes resistance to anticancer drugs. We also associate netrin-1 expression with 97 clinical progression and demonstrate that in various animal models, netrin-1 98 interference selectively impacts CSCs/TICs, thus potentiating sensitivity to 99 chemotherapy and anti-CTLA-4 monoclonal Antibody (mAb) immunotherapy. Finally, 100 we describe the integration of netrin-1 within the tumoral microenvironment and show 101 that the anti-netrin-1 inhibits tumor recruitment of a subtype of myeloid-derived 102 suppressor cells (MDSCs) called PMN-MDSCs. We provide functional evidence that 103 these immune cells promote the CSC phenotype, and consequent resistance to anti-104 cancer treatments.

105

107 **Results**:

108

109 Netrin-1 expression is associated with clinical progression and cancer stemness 110 in ovary and breast tumors.

Previous studies described high-level netrin-1 expression in a sizeable fraction 111 of human breast and ovarian cancers^{13,17,30}. Moreover, patients with metastasis at 112 diagnosis harbored primary tumors with greater netrin-1 expression than patients who 113 114 were metastasis-free¹³. To evaluate the impact of netrin-1 on prognosis, we analyzed 115 tumor expression of netrin-1 by quantitative-RT-PCR (q-RT-PCR) across a panel of 89 116 breast cancer patients treated with conventional chemotherapies, correlating netrin-1 117 expression data with clinical data. Clinical characteristics of the patients are presented 118 in **Supplementary Table 1**. Patients with tumors with elevated netrin-1 expression had 119 a significantly worse prognosis than patients with tumors with low netrin-1 expression. 120 The median survival of breast cancer patient was 9.8 years in the high netrin-1 group. 121 By contrast, the median survival was not yet reached at 15 years in the low netrin-1 122 group (Fig. 1A). Importantly, netrin-1 expression emerged as an independent 123 prognostic factor in multivariate analysis, together with the presence of metastasis at 124 diagnosis (Supp. Table 2). To better evaluate the association between netrin-1 125 expression and the risk of tumor relapse or progression, we evaluated the prognostic 126 value of netrin-1 tumor expression in the 67 patients with a localized disease, using 127 treatment-free survival as an endpoint in this retrospective analysis. As shown in 128 Figure 1B, median treatment-free survival was 9.9 years for patients with high netrin-129 1 tumor and was not reached for low netrin-1 group.

130

131 CSCs/TICs are hypothesized to play a major role in clinical progression and tumor recurrence^{22,31,32}. As shown in **Figure 1C**, netrin-1 expression was significantly 132 133 elevated in CSC marker CD44 high tumors vs CD44 low tumors (n=89). Similar data were obtained with other CSC markers (Supp. Fig. 1A). In parallel, using fresh tumoral 134 135 tissues from patients, we generated mammospheres to enrich stem-like 136 subpopulations. We observed that netrin-1 expression is lower in the overall bulk tumor 137 as compared to the paired mammospheres (Fig. 1D), indicating an enrichment of 138 netrin-1 in the subpopulation of cancer cells capable of regenerating spheres.

139 To analyze whether the results obtained in breast cancer could be extended to 140 other pathologies, we have investigated by immunohistochemistry (IHC) analysis the 141 expression of netrin-1 in ovarian carcinomas (Supp. Fig.1B). IHCs were performed on 142 biological samples taken at diagnosis in a cohort of 51 patients with high-grade serous 143 ovarian carcinomas without neoadjuvant therapy. Clinical characteristics of the 144 patients are presented in **Supplementary Table 3.** As shown in **Figure 1E**, patients 145 had a significantly poorer prognosis with increasing netrin-1 expression. Median 146 survivals were respectively 9.9, 5.7 and 3.3 years in the Negative. Low and High netrin-147 1 groups respectively. As shown in Figure 1F, median treatment-free survivals were 148 respectively 5.6, 3.1 and 1.8 years for patients with Negative, Low and High expression 149 of netrin-1 within the tumor. Similarly, netrin-1 was elevated in patients whose tumors 150 were resistant to platinum therapy (Fig. 1G) and increasing netrin-1 was concordant 151 with the CA-125, a circulating tumor marker known to correlate with tumor relapse 152 (Supplementary Fig. 1C). To determine the association of netrin-1 expression with 153 the CD44 stem marker, we performed q-RT-PCR analysis on a cohort of 22 patients 154 with high-grade serous ovarian carcinoma. As shown in **Figure 1H**, netrin-1 expression 155 was significantly higher in High CD44 tumors than in Low CD44 tumors. Taken 156 together, these expression data suggest that the level of netrin-1 is associated with 157 relapse and progression of breast and ovarian tumors and that netrin-1 is specifically 158 upregulated in CSCs/TICs compared to the bulk tumor.

159 The expression of netrin-1 in patient's tumors and its association with 160 CSCs/TICs led us to investigate the link between netrin-1 expression and stem 161 properties in a series of breast and ovarian cancer cell lines. With the idea that 162 chemoresistance is associated with stemness, we first identified Hs578t and SKBR7 163 as partially chemo-sensitive to Adriamycin (*i.e.*, ~20% of cells are still alive after 48h 164 of Adriamycin (Supp. Fig. 2A)) relative to other netrin-1 positive human cell lines 165 screened. Interestingly, following Adriamycin treatment, the surviving Hs578t and 166 SKBR7 cells were enriched for stem cell markers such as CD44 or CD49f, as well as 167 netrin-1 (Fig. 2A; Supp. Fig. 2B-C). We next investigated the ability of cells to form 168 spheres in breast and ovarian cell lines as a functional test to identify the existence of 169 a stem cell population. BT474, Hs578t, and OV90 form spheres (Supp. Fig. 2D) and 170 we observed a concordant elevation of stem cell markers and netrin-1, when compared 171 to the parental 2D cultures (Fig. 2B; Supp. Fig. 2E). To avoid the potential bias of analyzing CSCs enrichment after chemotherapy or sphere formation, we also performed an unbiased examination of netrin-1 expression in cells after sorting for stem cell markers positivity. Hs578t, SKBR7 breast cancer cells and OV90 ovary cancer cells have been sorted for respectively CD49f, ALDH or CD44 markers positivity associated with a clear increase of netrin-1 (**Fig. 2C**; **Supp. Fig. 2F-G**). This result highlights the association between netrin-1 and functional CSC markers in different tumor types.

179 In order to evaluate the involvement of netrin-1 in CSCs/TICs-mediated 180 treatment resistance, we first took advantage of a patient-derived xenograft (PDX) 181 immunocompromised mouse model in which a human triple negative breast tumor 182 sample -*i.e.*, HBC146- is grafted. This model was shown to be sensitive to a single 183 injection of chemotherapeutic agents, such as adriamycin and cyclophosphamide³³. 184 Human residual disease pathology includes a remission phase during which the tumor 185 is virtually undetectable, followed by a relapse phase in ~80% of the mice (Fig. 2D). 186 Netrin-1 expression was relatively low in the tumor bulk during the tumor growth phase 187 but was markedly elevated during the regression phase, returning to the basal level at 188 the time of relapse (**Fig. 2E**). Of interest, netrin-1 level appears fully correlated with the 189 presence of CD44 marker during all these stages, indicating a strong association of 190 netrin-1 with the stem character of the tumor cells (Fig. 2E). In order to link these higher 191 expression levels of netrin-1 and CD44 with an enrichment of CSCs during the tumor 192 regression phase more specifically, we dissected out untreated tumors or 193 chemotherapy-treated tumors prior to complete regression and performed CD44 194 analysis by flow cytometry or mammospheres forming assays. CD44^{high} cells were 195 enriched during the regression phase (Fig. 2F). Consistent with reports of CSC 196 resistance to chemotherapy, mammospheres formation occurred more frequently 197 when the tumors cells used were derived immediately following chemotherapy (Fig. 198 **2G**). We then proceeded to the enrichment of tumor-initiating cells from the untreated 199 tumor, either by CD44^{high} sorting or by mammospheres' cultures. Netrin-1 expression 200 was enriched in CD44^{high} cells (Supp. Fig. 2H). Paralleling our results in human breast 201 cancer samples, netrin-1 was less expressed in bulk tumors than in mammospheres 202 generated from these same tumors (Fig. 2H). Taken together, these data support the 203 view that in this breast cancer PDX model, netrin-1 is only weakly expressed in bulk

tumors but is more strongly expressed in a population of tumors cells resistant to thechemotherapeutic treatment that display elevated CSC markers.

206

207

7 Netrin-1 blockade induces cell death and delays relapse.

208 We and others groups have shown that netrin-1 up-regulation is a mechanism of escape from netrin-1 dependence receptor-induced apoptosis^{13–16,34}. In order to 209 210 investigate the potential survival role of netrin-1 in CSC, we first knocked down netrin-211 1 expression by siRNA in different cell lines. Silencing of netrin-1 is associated with 212 cell death induction in Hs578t as measured by a SubG1 assay (Fig. 2I). Moreover, this effect is increased when Adriamycin is combined as measured by cell viability (Fig. 213 214 **2J**). Along the same line, netrin-1 silencing impairs sphere formation ability of both 215 Hs578t and OV90 cells (Fig. 2K).

216 In order to achieve a pre-clinical and clinical development of targeting netrin-1, 217 a specific blocking monoclonal antibody (mAb) has been developped^{19,35}. When we 218 examined the effect of this antibody (mAb) in the previously described HBC146-PDX 219 model, we found that systemic delivery of the anti-netrin-1 mAb did not reveal any 220 significant tumor growth-inhibiting effect (Supp. Fig. 3A). This was consistent with the 221 low level of netrin-1 expressed in this tumor. However, evaluating this together with 222 combined chemotherapy, we observed a marked decrease of tumor regrowth at the 223 expected time of relapse observed in the chemotherapy-treated mice (Fig. 3A). While 224 the relapse-free median time in chemotherapy-treated animals was 56 days, this was 225 significantly delayed (*i.e.*, beyond the 75 days indicating the end of the experiment) in 226 the anti-netrin1 mAb-treated group (Fig. 3B). Consistent with a selective effect of 227 netrin-1 blockade on CSCs/TICs, the anti-netrin1 mAb treatment was associated with 228 an increase of cell death associated with a decrease in the number of cells able to form 229 spheres (Fig. 3C). This result was confirmed by immunohistochemical staining of 230 CD44, where anti-netrin-1 mAb-treated tumors showed a decrease of this stemness 231 marker in residual cancer cells (Fig. 3D).

To more definitively test the ability of netrin-1 blockade to impact mammary TICs in this model, we next performed serial transplant assays. HBC146 breast tumors were initially grafted in mice and treated twice weekly with either anti-netrin-1 mAb or a control IgG1. After 32 days, tumors were dissociated and regrafted into new recipient mice to evaluate tumor take in the absence of further treatment. As shown in **Figure** **3E**, anti-netrin-1 treatment is associated with a significant reduced tumor take in HBC146 serial transplantation assay. Importantly, similar results were obtained in a second breast cancer PDX model (BRE-012) where anti-netrin-1 treatment similarly failed to inhibit tumor growth (**Supp. Fig. 3B**) but was associated with a dramatic suppression of tumor take in serial transplantation assay (**Fig. 3F**).

To determine whether the results could be extended to other type of tumors, we 242 243 next performed a combination treatment using anti-netrin-1 with the standard of care 244 Carboplatin/Paclitaxel chemotherapy in the ovarian OV21 PDX model. Again, anti-245 netrin-1 mAb had no activity as a single agent (Supp. Fig. 3C) but combining anti-246 netrin-1 mAb with chemotherapeutic treatment triggered a better significant efficacy 247 than chemotherapy alone (Fig.3G). Similar effects were obtained with ovarian OV90 248 cells xenografts (Supp. Fig. 3D). This observation was associated with inhibition of 249 tumor take in a serial transplantation with anti-netrin-1 alone (Supp. Fig. 3E). This 250 was confirmed in mice engrafted with in high-grade serous ovarian carcinoma cell lines 251 OV90 and OVCAR3, where no inhibition of initial tumor growth was seen with anti-252 netrin-1 mAb (Supp. Fig.3F-G) but where strong inhibition of tumor take in a serial 253 transplantation assay was observed (Fig. 3H-I). Because in cancer settings the netrin-254 1 receptor involved appears to be mainly UNC5B, we have here investigated whether 255 the impact on CSCs was dependent on UNC5B. We used CRISPR interference to 256 invalidate UNC5B in HCC1937 breast cell line and grafted in mice and observed that 257 the absence of UNC5B completely altered the ability to initiate tumors (**Supp. Fig. 3H**). 258 Together, these data strongly support the idea that netrin-1/UNC5B blockade has a 259 specific impact on CSCs/TICs and prevents recurrence/resistance of breast and ovary 260 tumors after chemotherapy.

261

262 Netrin-1 blockade alleviates resistance to immune checkpoint inhibitor therapy.

Based on the impact on CSCs/TICs described here and the existing literature on cancer stemness, plasticity and resistance to immunotherapy^{24,36}, we next investigated whether netrin-1 blockade might also attenuate tumor resistance to immune checkpoint inhibitor (ICPI). For this we used the mouse mammary tumor cell line EMT6, which expresses netrin-1(**Supp. Fig. 4A**) and has some susceptibility to anti-CTLA-4^{37,38}. This model demonstrates a ~35% of tumor growth inhibition when treated with anti-CTLA-4 (**Supp. Fig. 4B**), though spaghetti curves indicate that it acts as an on/off model where mice may respond or not. About 60% of mice are nonresponders (**Supp. Fig. 4C**). This heterogeneity is highlighted by individual animalspecific cytokine secretion profiles, which reveals "cold" and "hot" animals in terms of immune response (**Supp. Table 4**).

274 Consistent with anti-CTLA4 activity, the level of regulatory T (T-reg) cells³⁹, was 275 downregulated in all mice, but less than 40% of mice demonstrated modulation of 276 tumor growth (Supp. Fig.4D). Anti-netrin-1 mAb was unable to significantly inhibit 277 tumor growth at all, when used as a single agent (Supp. Fig. 4E). However, the 278 combination of anti-netrin-1 with anti-CTLA-4 dramatically blocked tumor growth, and 279 strongly enhanced the long-term survival of mice bearing EMT6 tumors (Fig. 4A-B). 280 This increased response to the anti-CTLA-4 mAb induced by netrin-1 blockade was associated with increased secretion of pro-inflammatory cytokines such as IFNy 281 (**Supp. Fig. 4F**), suggesting a robust immune response^{24,40}. 282

283 In parallel studies, we analyzed the tumor immune infiltrate using both lymphoid 284 and myeloid panels by flow cytometry. We noticed an increase of CD8⁺ T lymphocytes 285 within tumors of mice treated with the combination of both antibodies, supporting the 286 notion that immune cell populations promote the enhanced anti-tumor effect observed 287 (Fig. 4C-D). In addition to T cell response, we analyzed the regulation of innate 288 immunity. A decrease in tumor associated macrophages was observed within tumors 289 of mice treated with the combination anti-netrin-1/anti-CTLA-4, supporting the view that 290 these immune cell populations promote the enhanced anti-tumor effect observed (Fig. 291 4E).

292

PMN-MDSCs participate to netrin-1 mediated cancer stem cell enrichment and resistance to immune checkpoint inhibitors.

295 To explore the underlying mechanisms linking immune infiltration and its 296 possible link with the anti-netrin-1 response, we next analyzed immune subpopulations 297 known to be involved in resistance to immune checkpoint inhibitors. We identified, in 298 the EMT6 engrafted model, a specific decrease in PMN-MDSCs⁴¹, a subtype of 299 myeloid-derived suppressor cells (MDSCs) derived from the 300 neutrophil/polymorphonuclear compartment (CD11b⁺ Ly6G⁺ Ly6C^{Interm}) within tumors 301 treated with anti-netrin-1 (Fig.5A). The same population was found to be down-302 regulated upon anti-netrin-1 mAb treatment in a spontaneous genetic breast cancer

303 model MMTV/NeuT. These mice spontaneously develop mammary tumors closed to
 304 human pathology⁴² (Fig.5B).

305 MDSCs have been reported to be able to control and protect cancer stem cell 306 populations⁴³. PMN-MDSCs are immature cells capable of inhibiting the functions of T 307 cells. They can accumulate during tumor growth and contribute to cancer 308 development⁴¹. We thus hypothesized that these PMN-MDSCs could link the effect of 309 netrin-1 interference on the tumor microenvironment and the resistance to treatments 310 mediated by CSCs/TICs. To analyze in vivo the MDSC function, we differentiate 311 immature myeloid cells into PMN-MDSCs. These immature myeloid cells were purified 312 from bone marrow of wild type mice and differentiated into PMN-MDSCs by treatment 313 with IL-6 and GM-CSF, that we reinjected into EMT6 tumor, treated with anti-netrin-1 314 and anti-CTLA-4. As a result, injection of PMN-MDSCs inhibit the effect of the combo-315 therapy highlighting the immuno-suppressive role of these cells (Fig. 5C). To analyze 316 if PMN-MDSCs can directly impact on CSCs, we evaluated their impact on CD49f^{High} 317 populations in a coculture model with EMT6 cells in which netrin-1 had been silenced. 318 As shown in Figure 5D, we observed that PMN-MDSCs sustained the CD49f⁺ stem 319 EMT6 cell population, an effect that was lost when netrin-1 was silenced. In vitro, PMN-320 MDSCs inhibit CD3⁺ T-cell proliferation and IFN_y production concomitant with the 321 activation of cytotoxic CD8⁺ lymphocytes, which clearly demonstrates their 322 immunosuppressive roles in this context (Supp. Fig.5A-B).

323 To determine whether the potentiation of the anti-CTLA-4 response by netrin-1 324 blockade was related to the impact of netrin-1 interference on CSCs/TICs, we analyzed 325 stemness markers in EMT6 tumors treated either with anti-netrin-1 mAb alone or in combination with anti-CTLA-4. By selecting CD45⁻ non-immune cells that were either 326 327 CD49f⁺ or CD326⁻ (murine stemness markers²⁶), we found that systemic anti-CTLA-4 328 treatment was associated with an enhancement of the stem cell populations (Fig. 5E; 329 **Supp. Fig. 5C**). This suggests that the anti-CTLA-4 antibody principally affects bulk 330 tumor cells, thus leaving residual tumors enriched for CSCs/TICs responsible for tumor 331 recurrence. Moreover, we quantified at the same time point PMN-MDSCs cells by 332 immunohistochemistry analysis (flow cytometry analysis was not possible due to a high 333 rate of necrosis linked to CTLA-4 cytotoxic activity). We show that Ly6G⁺ cells are 334 decreased both in the monotherapy with anti-netrin-1 mAb and in the combo with anti-335 CTLA-4, arguing in a protective role of these cell for CSCs (Fig. 5F).

336 We also assessed cancer stem cell function by performing a serial 337 transplantation assay comparing anti-netrin-1, anti-CTLA-4, or the combination. As 338 shown in Figure 5G, while treatment with anti-netrin-1 or its combination with anti-339 CTLA-4 completely abolished tumor take in serially transplanted mice, treatment with 340 anti-CTLA-4 only modestly inhibited tumor take. These data extend a literature which notes that ICPIs only partially impact cancer stem cell populations²⁴. Of major interest. 341 342 the combination of anti-CTLA-4 and anti-netrin-1 completely inhibits the enrichment in 343 these stem cell populations associated with anti-CTLA-4 antibody, making it a 344 promising strategy.

To further link our observations on cell subpopulations expressing stem cell 345 346 markers and CSC/TIC function, we sorted cells using CD49f and CD326 markers from 347 untreated EMT6 transplanted tumors and then transplanted them serially. As might be 348 predicted, we found that CD49f⁺ or CD326⁻ EMT6 cells were more prone to form tumors 349 than their CD49f and CD326⁺ counterparts (Supp. Fig. 5D-E). Moreover, CD49f⁺ cells 350 sorted from tumors treated with anti-netrin-1 mAb are losing their enhanced ability to 351 form new tumors in serial transplantation assays (Fig. 5H). To unravel the mechanism 352 behind, this effect we observed that while the In vitro treatment of EMT6 cells with anti-353 netrin-1 mAb leads to the decrease of CD49f⁺ cells (Supp. Fig. 5F), this decreased is 354 reverted when the general caspase inhibitor BOC was added (Supp. Fig. 5F)., supporting further the view that netrin-1 blockade impacts on CSCs by affecting cell 355 356 death, In order to demonstrate the role of the receptor in these experiments, we 357 invalidate UNC5B by shRNA in EMT6 and identified that the knock-down of UNC5B 358 altered the ability to initiate tumors in mice (Supp. Fig. 5G). Collectively, our data 359 support the view that the combination of ICPI and anti-netrin-1 is sensitizing grafted 360 tumors to ICPI by the impact of the anti-netrin-1 mAb on cancer stem cell populations 361 supported by the tumoral microenvironment.

- 362
- 363

Discussion 364

366 The present data identifies, for the first-time, netrin-1 as an independent 367 progression factor in breast and high grade serous ovarian cancers, associated with a 368 higher expression in CSCs/TICs. We show that netrin-1 interference may not only drive 369 tumor growth inhibition but may also be important in the prevention of tumor relapse 370 as well as in resistance to chemotherapy or immunotherapy. In support of this notion, 371 we identified that targeting netrin-1 in association with standard of care 372 chemotherapies induced a significant extension of the tumor-free survival (associated 373 with a delay in tumor relapse) in animal models of both breast and high grade ovarian 374 serous carcinoma. Similarly, we have shown that resistance to the ICPI anti-CTLA-4 375 could be alleviated by the association with the anti-netrin-1 mAb.

376 A challenging question remains; to understand the molecular mechanisms 377 inhibited or unleashed upon netrin-1 blockade that confer relapse delay and/or alleviate 378 resistance. We propose here that netrin-1 may play a specific role in CSCs/TICs since 379 (i) netrin-1 is often specifically more expressed in CSCs/TICs compared to bulk tumor 380 cells and (ii) netrin-1 blockade appears to reduce CSCs/TICs -i.e., monitored using 381 both functional assays or markers. Although netrin-1 was initially considered as a 382 guidance cue, several recent published works seem to describe a role of netrin-1 in 383 induced pluripotent stem cells, embryonic stem cells and naïve pluripotency^{44–46}. The 384 link between normal and embryonic stemness has not been extended to cancer 385 "stemness," a prior in vitro study reported a possible role of netrin-1 in glioblastoma 386 CSCs⁸. Based on an initial work from our lab, netrin-1 has been described as a survival 387 factor in iPSC and ES, and acts in part by blocking the death induced by the dependence receptor UNC5B⁴⁴. More recent work suggests that netrin-1 also links the 388 389 Wnt and MAPK pathways⁴⁵. Whether the biology behind "normal" stemness and 390 CSCs/TICs relies on similar mechanisms should now be examined. Similarly, whether 391 the reduction of CSCs/TICs associated with netrin-1 blockade seen in this manuscript 392 is related to increased cell death or "lineage" commitment remains to be investigated. 393 Nonetheless, our work, for the first time, links netrin-1 to breast and ovarian cancer 394 progression and suggests it to be a *bona fide* clinical target. Based on these studies, 395 a phase 2 clinical trial (https://clinicaltrials.gov/ct2/show/NCT04652076) was started in

396 late 2020 combining anti-netrin-1 and standard of care chemotherapy as well as397 immunotherapy.

398 The data obtained with the combination of ICPI with the anti-netrin1 mAb are of 399 specific importance taken into account the currently limited clinical activity of ICPI in 400 breast cancer ^{37,38}. The data presented here support the view that the suppression of 401 ICPI resistance by netrin-1 blockade is associated with its impact on CSCs/TICs. 402 However, we cannot yet exclude others additional mechanisms. Moreover, previous 403 works have described the role of netrin-1 in the migration or the survival of immune cells such as macrophage⁴⁻⁷ or CD4⁺ cells⁴⁷ during inflammatory arthritis or obesity. 404 MDSCs have already been reported to modulate the response to ICPI drugs^{41,48}. 405

406 PMN-MDSCs have already been shown to be capable of sustaining tumor 407 progression⁴¹. In our investigations, we identify that netrin-1 blockade silences these 408 cells. We propose a novel pro-tumoral effect of immune cells, sustain CSCs/TICs and 409 thus may indirectly affect resistance to treatment. This proposed mechanism is 410 consistent with a mechanism in which the potentiation of ICPI by netrin-1 blockade 411 does not necessarily occur through direct reinforcement of immune effector cells but 412 rather through an indirect effect on CSCs/TICs. We thus propose that netrin-1 413 produced by cancer cells sustains PMN-MDSC which in turn sustains CSCs/TICs that 414 contribute to both recurrence and to resistance to chemotherapies and ICPIs.

However it is fair to say it probably includes additional players of the tumor microenvironment more generally impacting on tumoral plasticity such as CAFs⁴⁹, additional immune cells, endothelial cells, and extracellular matrix but also more generally the cellular stress reported in tumoral and stromal cells²⁵. Whether and how netrin-1 might interact with these additional players remains an open question.

421 Materials and Methods:

422

423 Ethics, Human tumors samples and biological annotations:

424 The usage of all patient tumor samples was supported according to French regulations 425 on the protection of biomedical investigation subjects. The collection of human 426 samples was realized with primary female breast tumors from patients treated at the 427 Centre Léon Bérard, conserved in the Centre des Ressources Biologiques (CRB) of 428 the Centre Léon Bérard. Informed consent was obtained from all patients, and the study use samples which were approved by the ethics committee of the institutions. 429 430 Fresh samples were addressed for mammosphere formation experiments, frozen 431 tissues for q-RT-PCR analysis and fixed tissues to immunohistochemistry.

432

433 Cell culture

434 All the cell lines were obtained from ATCC. Murine mammary carcinoma EMT-6 cells were cultured in Eagle's Minimum Essential Medium (EMEM, ATCC) complemented 435 with 10% of Serum Foetal Bovine (FBS, Gibco) and antibiotics (Streptomycin and 436 437 Penicillin). They were cultured in Dulbecco's Modified Eagle's Medium (DMEM) for 438 SKBR7 and Hs578t or RPMI for BT474, T47D, MDA-MB468, OV90 and OVCAR3, 439 complemented with 10% FBS (Gibco) and antibiotics. HCC1937 cell line was cultured 440 in RPMI complemented with 10% FBS (Gibco), 2% Na Bicarbonate, 1% Na Pyruvate, 441 1% HEPES and antibiotics. Cells were maintained in culture at 37°C under humidified 442 atmosphere consisting of 20% O₂ and 5% CO2. For the CRISPRi mediated silencing 443 of hUNC5B in HCC1937 cell line, a small guide RNA (sgRNA) targeting hUNC5B 444 promoter designed using the sgRNA designer was tool 445 (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design-crisprai). А 446 control sgRNA targeting Firefly luciferase was also designed. Sequences are as 447 follows: sgUNC5B: 5'- GCGCGCCTCTCGGAGCCCCG -3' and sgLUC: 5'-CGCCCACGGCCCTTCCCGGG-3'. Forward and reverse sgRNA-coding oligos with 448 449 Esp3I linkers were ordered from Eurofins Genomics and then annealed by incubation 450 for 3 min at 90°C and 15 min at 37°C and ligated in pLV hU6-sgRNA hUbC-dCas9-KRAB-T2a-Puro ⁵⁰ Insertion of sgRNA was confirmed by sequencing. The use of pLV 451 hU6-sgRNA hUbC-dCas9-KRAB-T2a-Puro allows for the simultaneous expression of 452 453 dCas9-KRAB and sgRNA and for selection for puromycin resistance. pLV hU6-sgRNA 454 hUbC-dCas9-KRAB-T2a-Puro was a gift from Charles Gersbach (Addgene plasmid # 455 71236; http://n2t.net/addgene:71236 ; RRID:Addgene_71236). For the shRNA 456 mediated KO of mUNC5B in EMT6 cell line, a pLKO.1-puro vector encoding shRNA 457 targeting mUNC5B (Clone ID: TRCN0000072079, shRNA sequence: 5'-458 CGCCTACATCGTAAAGAACAA-3') was purchased from Sigma-Aldrich. Production of 459 VSV-g pseudotyped lentivirus was made as described previously.

460

461 *In vivo* mice models and materials:

462 Human anti-netrin-1 monoclonal antibodies (anti-netrin1) were supplied by Netris 463 Pharma (Lyon, France). Female Swiss nude mice, 6-weeks old, were purchased from 464 Charles River laboratories (Les Oncins, France) and maintained in specific pathogen-465 free conditions (Anican, Lyon, France) and stored in sterilized filter-topped cages. Their care and housing were in accordance with institutional European guidelines as put 466 forth by the CECCAP local Ethical Committee (C2EA-15, CLB_2014_001; 467 468 CLB 2014 012; CECCAPP CLB 2016 017). The human human triple negative breast cancer patient derived xenograft PDX HBC146 was established and 469 characterized for tumor recurrence by Marangoni *et al*^{β 3}. The human triple negative 470 breast cancer patient derived xenograft BRE-012 was established by the IMODI 471 consortium(https://imodi-cancer.org/fr/). PDX-OV21 human high grade serous ovarian 472 473 carcinoma model has been established by Curie Institute and previously published⁵¹.

For PDX experiments, mice were grafted as described by Marangoni et al, 2007^{24} . Tumor growth was evaluated by measurement of two perpendicular diameters of tumors with a caliper twice per week. Individual tumor volumes were calculated as V = (length*width²)/2., a being the largest diameter, b the smallest. When tumors reached a volume of 300mm³, the mice were randomly separated in two groups and submitted 479 anti-netrin-1 or Control alone, or in combination to standard or care chemotherapy: 480 Adriamycin, 2 mg/kg (Doxorubicin, Teva Pharmaceuticals), and cyclophosphamide, 481 100 mg/kg (Endoxan, Baxter) were inoculated with a single i.p. injection or Carboplatin 482 66 mg/Kg (generic provided by Hospira) and Paclitaxel 30 mg/Kg (generic provided by 483 Hospira), i. p. injection once a week Anti-netrin-1 mAb or IgG1 control were i.p. injected 484 with 10mg/kg twice per week for the rest of the experiment. Animal work has been done by Netris pharma, Institut Curie or the Laboratoire des Modèles Tumoraux (LMT). 485 486 5 x 10⁶ OV90 and OVCAR3 cells were subcutaneously inoculated with Matrigel 487 (Corning) in the dorsal flank of 8-week-old female Swiss/Nude mice. The mice were 488 housed under specific pathogen free (SPOF) conditions (Anican, Lyon, France). Tumor 489 volumes were evaluated approximately every 3 days after initial detection using 490 calliper⁵²

491 For experiments of immune checkpoints inhibitors, eight-week-old (20-22 g body 492 weight) female BalbC/J mice were obtained from Janvier Labs. 1 x 10⁶ EMT-6 cells 493 were subcutaneously inoculated in the dorsal flank of 8-week-old female BALB/cJ wild-494 type mice. The mice were housed under specific pathogen free (SPF) conditions as previously described (Anican, Lyon, France)⁵³. Tumor volumes were evaluated 495 approximately every 2 days after initial detection using calliper⁵². Based on initial intra-496 497 group homogeneity of tumor size, four different treatments have been administrated: 498 IgG1 control isotype (Netris Pharma), anti-netrin-1 mAb (NP137, Netris Pharma) 499 combination of anti-CTLA-4 mAb (BioXCell, Clone 9H10) with IgG1 and combination 500 of anti-CTLA-4 with anti-netrin-1 mAb. The treatments were ip. administered respecting the following dose: 20 mg/kg anti-CTLA-4, 10 mg/kg anti-netrin-1 and 10 mg/kg mice 501 IgG1 on days 8, 11 and 14 after the implant. Mice were sacrificed when individual 502 503 tumours reached a predefined endpoint (1000-2000 mm³).

504 For the injection of MDSCs cells, 500 000 EMT6 breast cancer cells were mixed with 505 50 000 PMN-MDSCs (ratio 10:1) produced *in vitro* and were inoculated in the dorsal 506 flank of 8-week-old female BALB/cJ wild-type mice. Subsequently mice were 507 intratumorally inoculated with PMN-MDSC at day 8 and 13. 508

509 Serial transplantation, tumor-initiating cell sorting and mammosphere culture:

Tumors were dissociated with a collagenase (Sigma Aldrich) / DNAse (Life 510 511 Technologies) for 45 minutes at 37°C, submitted to red blood cell lysis (Miltenyi 512 Biotech) and passed through a 30 µm filter to obtain a single cell suspension. Cell 513 viability has been evaluated by trypan blue count under the microscope to ensure the 514 engraftment of the same number of cells with a similar viability in the different 515 conditions of treatment, residual cells were after analyzed and sorted by FACS or 516 submitted to mammosphere formation. FACS sorting was performed on a BD 517 FACSDiVa. Anti-CD44-PE (BD system) was used to count and sort CD44^{high} cells and 518 dead cells were counted with propidium iodine staining. ALDEFLUOR (Stemcell 519 Technologies) was used to count and sort ALDH^{high} cells and dead cells were counted 520 with propidium iodine staining. Mammosphere enrichments were obtained by culturing 521 the cells on 6 wells ultra-low attachment dishes in mammocult medium (Stem cell 522 technologies). Cells were allowed to form clonal mammospheres for 7 to 10 days of 523 culture. For mammosphere forming assays, viable singlets were sorted by FACS and 524 directly plated on 96 wells ultra-low attachment dishes at the density of 1000 cells per 525 well in mammocult medium. Mammospheres were counted after 7 days of culture. To 526 analyze spheres passages, 7 days spheres were dissociated and 1000 cells per well 527 were again plated in the conditions mentioned above and counted after 7 days of 528 culture.

529

530 Immunohistochemistry:

Immunohistochemistry analysis was performed on 4-μm-thick sections of paraffin
embedded mouse tissues stained with anti-CD44 (156-3C11, Thermo Fisher) or antinetrin-1 (CPA2389, Cohesion Biosciences) or anti-Ly-6G (E6Z1T- Rabbit mAb #87048
Cell signaling) dilution 1/500. Expressions are revealed by brown DAB staining. Nuclei
are counterstained by hematoxylin (blue).

537 Flow cytometry analysis:

538 EMT6 subcutaneous tumors were mechanically disaggregated in RPMI-1640 medium 539 5% FBS and enzymatically digested with 1 mg/ml of collagenase I and 40 U/ml of 540 DNAse I (Sigma Aldrich) for 30 min at 37 °C. Cells were passed through a 70 µm-cell 541 strainer and filtered. After RBC lysis, cells were passed through a 30 µm-cell strainer and filtered. Cell pellets were resuspended in HBSS (Gibco) before counting. 2 x 10⁶ 542 543 cells were stained for the viability with Zombie Agua (Biolegend) for 30 minutes at 4°C. Cells were washed and incubated with Fc Block purified anti-mouse CD16/CD32 544 545 (Clone 93, Biolegend), followed by staining with mouse anti-CD4 (RM4-5, Brilliant 546 Violet 785), anti-CD19 (6D5, Brilliant Violet 605), anti-NKp46 (29A1.4, PE), anti-CD45 (30-F11, Alexa Fluor 700), anti-CD25 (PC61, APC), anti-CD69 (H1.2F3, FITC), anti-547 IA-IE (M5/114.15.2, BV421), anti-Ly6C (HK1.4, APC-Cy7) anti-Ly6G (1A8, FITC), 548 CD11b (M1/70, BV785) and F4/80 (BM8, APC) from Biolegend and anti-CD3 (clone 549 500A2, PerCP-eFluor 710, anti-CD8b (eBioH35-17.2, eFluor 450), anti-CD44 (IM7, 550 551 PerCP-Cy5.5), anti-CD326 (G8.8, PE-Dazzle 594) from eBioscience, anti-CD49f 552 (GoH3, eFluor 450) from Life Technologies, for 45 min at 4 °C. Cells were fixed and permeabilized with the Foxp3 transcription factor staining buffer set (eBiosciences), 553 and overnight stained for 30 min at 4 °C for IFN γ (XMG1.2, PE, Biolegend) and with 554 555 anti-Foxp3 (FJK-16s, PE-Cyanine7, eBioscience). Analyses were performed with 556 Fortessa HTS flow cytometer (BD Biosciences). Data were analyzed with FlowLogic 557 Version 6 (Miltenyi Biotec, Germany). All gates were defined using fluorescence minus 558 one control (FMO).

559

560 **Cell isolation**

561 CD3⁺ T-cells were isolated from the Lymph nodes of 7-week-old BALB/cJ wild-type 562 mice. The separation was performed by using Pan T Cell Isolation Kit (Miltenyi Biotech) 563 and MACS column. Purity of cell population were evaluated by flow cytometry. CD3⁺ 564 T-cells were cultivated *in-vitro* for 3-days with Anti-CD3 and Anti-CD28 (Dynabeads, 565 Gibco) and IL-2 (10 ng/ml). Immature myeloid cells were purified from bone marrow of 566 7-week-old BALB/cJ wild-type mice. Red blood cells (RBC) were lysate with ACK 567 (Ammonium-Chloride-Potassium) Lysing Buffer, cells were filtered through a 70 µm-568 cell strainer. Immature myeloid cells were cultivated *in-vitro* for 4-days with GM-CSF 569 and IL-6 (40ng/ml) to differentiate them. T-cells and PMN-MDSCs were cultivated in 570 RPMI complemented with 10% FBS (Gibco), 1% Na Bicarbonate, 1% Na Pyruvate, 571 1% L-glutamine (Gibco), 1% HEPES, 0.05 mM 2-mercaptoethanol (Gibco) and 572 antibiotics.

573

574 **Proliferation viability and cell death assays**

575 CD3⁺ T-cells were labeled with cell trace violet (CTV, Invitrogen) as follows: 10⁶ cells 576 were incubated with 1 µM of CTV in PBS for 20 min at 37°C, 5% CO2. 5-volume of 577 culture medium were added, and cells were left at 37°C 5% CO2 in culture medium for 578 additional 5 min. Cells were analyzed by flow cytometry for the proliferation baseline 579 or co-cultured with PMN-MDSCs. WST1 assay was used according to the 580 manufacturer (Cell proliferation Reagent, Roche) to measure cell viability. Cells are 581 fixed in 70% Ethanol and stained with Propidium iodide (PI). SubG1 population is 582 further identified by flow cytometry.

583

584 **IFN-γ Production**

585 Activated CD3⁺ T-cells were stimulated with phorbol myristate acetate (PMA), 586 Ionomycin, Brefeldin (Biolegend) and Monensin (Biolegend) for 4 hours at 37°C, 5% 587 CO2 in complete culture medium. IFN γ released by CD8⁺ T- cells was evaluated by 588 flow cytometry.

589

590 **Co-culture**

591 To test the immunosuppressive activity of PMN-MDSCs were co-cultured with purified 592 CD3⁺ T-cells for 3 days at different ratio (MDSCs: CD3⁺ T-cells, 1:1, 1:4) in 96-well 593 plate. To analyze the effect PMN-MDSCs on stemness were co-cultured with EMT6-594 control vector EMT6-Sh netrin-1 in a ratio 1:10 (MDSCs: Tumoral cells) for 24 hours in 595 24 well plate. CD49f^{high} cells were analyzed by flow cytometry.

596

597 **Cytokine analysis:**

To measure the systemic release of IFN γ , fresh stabilized whole blood was drawn from BALB/cJ wild-type mice 16h hours after 1st, 2nd and 3rd treatments. Plasma was separated and used to measure the protein levels of IFN γ with the Mouse IFN-gamma Quantikine ELISA Kit (R&D System, Minnesota, USA) according to the manufacturer's instructions.

603 For cytokines array analysis, 31 cytokines and chemokines were analyzed 18 hours 604 after the 2nd CTLA-4 treatment, using the multiplex assays manufactured by Meso Scale Discovery (MSD; Gaithersburg, MD). EMT6 subcutaneous tumors were 605 606 mechanically disaggregated in 500µl of RPMI-1640 medium (in absence of FBS) for 607 500 mg of tumor. The cytokines and chemokines analyzed accordingly to the 608 manufacturer recommandations and were IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-15, IL-16, IL-17A, IL-17A/F, IL-17C, IL-17E/IL-25, IL-21, IL-22, IL-23, 609 IL27p28/IL30, IL-31, IL-33, IFN γ, MIP-1α, MIP-2, MIP-3α, IP-10, MCP-1, KC/GRO, 610 TNF- α , TGF- β 2, TGF- β 3, TGF- β 1. Analyses were performed using the software 611 612 Discovery workbench 4.0.

613

614 **Immunoblots**:

615 Confluent cells were washed with cold PBS and scrapped in lysis buffer (Tris 10 mM pH 7.6; SDS 5; Glycerol 10%; Triton X-100 1%, DTT 100mM). After sonication proteins 616 were dosed using Pierce 660nm Protein Assay Reagent (Thermo Fisher Scientific) and 617 618 after loading on 4%-15% SDS-polyacrylamide gels (Bio-Rad) transferred to 619 nitrocellulose membranes using Trans-Blot Turbo Transfer (Bio-Rad). Membranes 620 were blocked for 1 hour at room temperature with 5% of nonfat dried milk for the Netrin-621 1 and with 5% of BSA and 1% of nonfat dried milk for UNC5B. Staining was performed 622 overnight with primary antibody: anti-netrin1 antibody (Ab126729, Abcam) and anti-623 UNC5B (D9M7Z, Cell signaling technology). After washing, membranes were

incubated with secondary antibody, goat anti-rabbit coupled with HRP for 1 hour at
 room temperature. West Dura Chemiluminescence System (Pierce) was used to
 intensify the signal. Imaging was performed using Chemidoch Touch (Bio-Rad). Full
 membrane westernblots were included in the supplementary material.

628

629 **RNA expression analysis**:

Total mRNAs were extracted from tissues or cells using Nucleospin RNAII kit
(Macherey-Nagel) and 1 µg was reverse transcribed using the iScript cDNA Synthesis
kit (Bio-Rad). Real-time quantitative RT–PCR was performed on a LightCycler 2.0
apparatus (Roche, Meylan, France) using the Light Cycler Master Taqman kit (Roche,
Meylan, France). Oligonucleotide sequences are available upon request.

635

636 **Statistical analysis:**

To test netrin-1 expression, cohorts were dichotomized based on Netrin-1 median expression. Survival curves were produced by the Kaplan-Meier method on Prism (GraphPad Software, San Diego, California). Data were analyzed using a Mantel-Cox test. n defines the total number of replicates. All statistical tests were two-sided. The multivariate analysis was realized with COX model in the R software. Kaplan-Meier curves of disease-free survival of overall survival were analyzed with a Gehan-Breslow-Wilcoxon Test in the GraphPad Prism software.

644 For mice experiments, statistical methods were not used to predetermine necessary 645 sample size, but sample size was chosen based on pilot experiments which applying 646 appropriate statistical test could return significant results. Tumor growth with time was 647 analyzed by two-way ANOVA with Bonferroni correction. Survival curves were 648 analyzed using the Log-rank (Mantel-Cox) test. Flow cytometry data are reported as 649 mean±s.e.m. in case of representative experiment; the pool of different experiments is 650 expressed as a *ratio* of the control group of the single experiment. Statistical analysis 651 was performed by Mann-Whitney test (GraphPad).

655 Acknowledgments:

We thank Dwayne Stupack for correction and helpful discussion, the LMT platform for the high quality of the animal work. This work was supported by Netris Pharma and institutional grants from CNRS (PM, BG), University of Lyon (PM), Centre Léon Bérard (PM) and from the Ligue Contre le Cancer (PM), INCA (PM), Euronanomed (PM, BG), ANR (PM), BMS Foundation for Immunotherapy (PM). AR received a 4th year fellowship from Ligue contre le Cancer.

662

663 **Conflict of interest:**

BD, CV, JL, DN, DG, SD, AB and PM declare to have a conflict of interest as
respectively employees (BD, DN, DG, CV, JL and SD) and shareholders (AB and PM)
of NETRIS Pharma.

667

668 Author contributions:

- BD, ARR, CV, RC, RB, AP and JL performed the cell experiments and *in vitro* data;
- 670 BD, ARR, MH, PV, DN, MR, IG, FN and PAL performed mice experiments;
- 671 CV, RB, AP and DG produced the lentiviruses and CRISPR and Sh cell lines;
- BD, AF, IT and NG made anatomopathological analysis;
- 673 NR performed bioinformatical analysis;
- NG, IT, IRC, EML, CG, STE and MDS provided human samples and cell lines;
- 675 BD, ARR and SC performed statistical analysis;
- BD, SD, DD, EM, KJM, BG, PM and AB have participated to the research design and
- 677 to write the paper.
- 678

679 Data Availability statement:

- 680 Data sharing not applicable to this article as no datasets were generated or analysed during the
- 681 current study.

682 **References:**

683 Serafini T, Colamarino SA, Leonardo ED, Wang H, Beddington R, Skarnes 1 684 WC et al. Netrin-1 Is Required for Commissural Axon Guidance in the Developing 685 Vertebrate Nervous System. Cell 1996; 87: 1001-1014. 686 Chédotal A. Roles of axon guidance molecules in neuronal wiring in the 2 687 developing spinal cord. Nat Rev Neurosci 2019; 20: 380-396. 688 3 Mehlen P, Delloye-Bourgeois C, Chédotal A. Novel roles for Slits and netrins: 689 axon guidance cues as anticancer targets? Nat Rev Cancer 2011; 11: 188-197. Mehlen P, Bredesen DE. Dependence receptors: from basic research to drug 690 4 691 development. Sci Signal 2011: 4: mr2. 692 Bongo JB, Peng DQ. The neuroimmune guidance cue netrin-1: a new 5 therapeutic target in cardiovascular disease. J Cardiol 2014; 63: 95-98. 693 694 van Gils JM, Derby MC, Fernandes LR, Ramkhelawon B, Ray TD, Rayner KJ 6 695 et al. The neuroimmune guidance cue netrin-1 promotes atherosclerosis by inhibiting 696 the emigration of macrophages from plagues. Nat Immunol 2012; 13: 136-143. Ramkhelawon B, Hennessy EJ, Ménager M, Ray TD, Sheedy FJ, Hutchison S 697 7 698 et al. Netrin-1 promotes adipose tissue macrophage retention and insulin resistance 699 in obesity. Nat Med 2014; 20: 377-384. 700 8 Sanvoranart T, Supokawej A, Kheolamai P, U-pratya Y, Poungvarin N, 701 Sathornsumetee S et al. Targeting Netrin-1 in glioblastoma stem-like cells inhibits 702 growth, invasion, and angiogenesis. Tumor Biol 2016; 37: 14949-14960. 703 Barnault R, Verzeroli C, Fournier C, Michelet M, Redavid AR, Chicherova I et 9 704 al. Hepatic inflammation elicits production of proinflammatory netrin-1 through 705 exclusive activation of translation. Hepatology 2022; : hep.32446. Fazeli A, Dickinson SL, Hermiston ML, Tighe RV, Steen RG, Small CG et al. 706 10 707 Phenotype of mice lacking functional Deleted in colorectal cancer (Dcc) gene. Nature 708 1997; **386**: 796-804. 709 Leonardo ED, Hinck L, Masu M, Keino-Masu K, Ackerman SL, Tessier-11 710 Lavigne M. Vertebrate homologues of C. elegans UNC-5 are candidate netrin 711 receptors. Nature 1997; 386: 833-838. 712 Gibert B, Mehlen P. Dependence Receptors and Cancer: Addiction to Trophic 12 713 Ligands. Cancer Research 2015; 75: 5171–5175. Fitamant J, Guenebeaud C, Coissieux M-M, Guix C, Treilleux I, Scoazec J-Y 714 13 715 et al. Netrin-1 expression confers a selective advantage for tumor cell survival in metastatic breast cancer. Proceedings of the National Academy of Sciences 2008; 716 717 **105**: 4850–4855. 718 14 Delloye-Bourgeois C, Fitamant J, Paradisi A, Cappellen D, Douc-Rasy S, 719 Raquin M-A et al. Netrin-1 acts as a survival factor for aggressive neuroblastoma. J 720 Exp Med 2009; 206: 833-847. 721 Delloye-Bourgeois C, Brambilla E, Coissieux M-M, Guenebeaud C, Pedeux R, 15 722 Firlej V et al. Interference with netrin-1 and tumor cell death in non-small cell lung 723 cancer. J Natl Cancer Inst 2009; 101: 237-247. 724 Paradisi A, Creveaux M, Gibert B, Devailly G, Redoulez E, Neves D et al. 16 Combining chemotherapeutic agents and netrin-1 interference potentiates cancer cell 725 726 death. EMBO Mol Med 2013: 5: 1821-1834. Grandin M, Meier M, Delcros JG, Nikodemus D, Reuten R, Patel TR et al. 727 17 728 Structural Decoding of the Netrin-1/UNC5 Interaction and its Therapeutical Implications in Cancers. Cancer Cell 2016; 29: 173-185. 729 730 18 Kryza D, Wischhusen J, Richaud M, Hervieu M, Sidi Boumedine J, Delcros J

731 et al. From netrin-1-targeted SPECT / CT to internal radiotherapy for management of 732 advanced solid tumors. EMBO Mol Med 2023; 15: e16732. 733 19 Cassier P, Eberst L, Garin G, Courbebaisse Y, Terret C, Robert M et al. A first in human, phase I trial of NP137, a first-in-class antibody targeting netrin-1, in 734 patients with advanced refractory solid tumors. Annals of Oncology 2019; 30: v159. 735 736 Cassier PA, Navaridas R, Bellina M, Rama N, Ducarouge B, Hernandez-20 737 Vargas H et al. Netrin-1 blockade inhibits tumour growth and EMT features in 738 endometrial cancer. Nature 2023. doi:10.1038/s41586-023-06367-z. 739 Lengrand J, Pastushenko I, Vanuytven S, Song Y, Venet D, Sarate RM et al. 21 740 Pharmacological targeting of netrin-1 inhibits EMT in cancer. Nature 2023. 741 doi:10.1038/s41586-023-06372-2. 742 Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and 22 743 cancer stem cells. Nature 2001; 414: 105-111. 744 Yeung TM, Gandhi SC, Wilding JL, Muschel R, Bodmer WF. Cancer stem 23 745 cells from colorectal cancer-derived cell lines. Proc Natl Acad Sci USA 2010; 107: 746 3722-3727. 747 Miao Y, Yang H, Levorse J, Yuan S, Polak L, Sribour M et al. Adaptive 24 748 Immune Resistance Emerges from Tumor-Initiating Stem Cells. Cell 2019; 177: 749 1172-1186.e14. Lu H, Xie Y, Tran L, Lan J, Yang Y, Murugan NL et al. Chemotherapy-induced 750 25 S100A10 recruits KDM6A to facilitate OCT4-mediated breast cancer stemness. 751 752 Journal of Clinical Investigation 2020. doi:10.1172/JCI138577. 753 Batlle E, Clevers H. Cancer stem cells revisited. Nat Med 2017; 23: 1124-26 754 1134. 755 Ye L, Lin C, Wang X, Li Q, Li Y, Wang M et al. Epigenetic silencing of SALL 2 27 756 confers tamoxifen resistance in breast cancer. EMBO Mol Med 2019; 11. 757 doi:10.15252/emmm.201910638. 758 Arfaoui A, Rioualen C, Azzoni V, Pinna G, Finetti P, Wicinski J et al. A 28 759 genome-wide RNA i screen reveals essential therapeutic targets of breast cancer 760 stem cells. EMBO Mol Med 2019; 11. doi:10.15252/emmm.201809930. Junttila MR, de Sauvage FJ. Influence of tumour micro-environment 761 29 heterogeneity on therapeutic response. Nature 2013; 501: 346-354. 762 Papanastasiou AD, Pampalakis G, Katsaros D, Sotiropoulou G. Netrin-1 763 30 764 overexpression is predictive of ovarian malignancies. Oncotarget 2011; 2: 363-367. Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M et al. 765 31 ALDH1 is a marker of normal and malignant human mammary stem cells and a 766 predictor of poor clinical outcome. Cell Stem Cell 2007; 1: 555-567. 767 Charafe-Jauffret E, Ginestier C, Iovino F, Tarpin C, Diebel M, Esterni B et al. 768 32 769 Aldehyde dehydrogenase 1-positive cancer stem cells mediate metastasis and poor 770 clinical outcome in inflammatory breast cancer. Clin Cancer Res 2010; 16: 45-55. 771 Marangoni E, Lecomte N, Durand L, de Pinieux G, Decaudin D, Chomienne C 33 772 et al. CD44 targeting reduces tumour growth and prevents post-chemotherapy 773 relapse of human breast cancers xenografts. Br J Cancer 2009; 100: 918-922. 774 Dumartin L, Quemener C, Laklai H, Herbert J, Bicknell R, Bousquet C et al. 34 775 Netrin-1 mediates early events in pancreatic adenocarcinoma progression, acting on 776 tumor and endothelial cells. Gastroenterology 2010; 138: 1595-1606, 1606.e1-8. 777 Grandin M, Meier M, Delcros JG, Nikodemus D, Reuten R, Patel TR et al. 35 778 Structural Decoding of the Netrin-1/UNC5 Interaction and its Therapeutical 779 Implications in Cancers. Cancer Cell 2016; 29: 173-185. 780 Dongre A, Rashidian M, Reinhardt F, Bagnato A, Keckesova Z, Ploegh HL et 36

- *al.* Epithelial-to-Mesenchymal Transition Contributes to Immunosuppression in Breast
 Carcinomas. *Cancer Res* 2017; **77**: 3982–3989.
- 37 Bayraktar S, Batoo S, Okuno S, Glück S. Immunotherapy in breast cancer. J
 784 Carcinog 2019; 18: 2.
- 785 **38** Polk A, Svane I-M, Andersson M, Nielsen D. Checkpoint inhibitors in breast 786 cancer – Current status. *Cancer Treatment Reviews* 2018; **63**: 122–134.
- 787 39 Read S, Greenwald R, Izcue A, Robinson N, Mandelbrot D, Francisco L *et al.*788 Blockade of CTLA-4 on CD4 ⁺ CD25 ⁺ Regulatory T Cells Abrogates Their Function
 789 In Vivo. *J Immunol* 2006; **177**: 4376–4383.
- Ni L, Lu J. Interferon gamma in cancer immunotherapy. *Cancer Med* 2018; 7:
 4509–4516.
- 792 41 Zhou J, Nefedova Y, Lei A, Gabrilovich D. Neutrophils and PMN-MDSC: Their
 793 biological role and interaction with stromal cells. *Seminars in Immunology* 2018; 35:
 794 19–28.
- Guy CT, Webster MA, Schaller M, Parsons TJ, Cardiff RD, Muller WJ.
- Expression of the neu protooncogene in the mammary epithelium of transgenic mice
 induces metastatic disease. *Proceedings of the National Academy of Sciences* 1992;
 89: 10578–10582.
- Peng D, Tanikawa T, Li W, Zhao L, Vatan L, Szeliga W *et al.* Myeloid-Derived
 Suppressor Cells Endow Stem-like Qualities to Breast Cancer Cells through
- IL6/STAT3 and NO/NOTCH Cross-talk Signaling. *Cancer Res* 2016; **76**: 3156–3165.
 44 Ozmadenci D, Féraud O, Markossian S, Kress E, Ducarouge B, Gibert B *et al.*Netrin-1 regulates somatic cell reprogramming and pluripotency maintenance. *Nat*
- 804 *Commun* 2015: **6**: 7398.
- Huyghe A, Furlan G, Ozmadenci D, Galonska C, Charlton J, Gaume X *et al.*Netrin-1 promotes naive pluripotency through Neo1 and Unc5b co-regulation of Wnt
 and MAPK signalling. *Nat Cell Biol* 2020; **22**: 389–400.
- Renders S, Svendsen AF, Panten J, Rama N, Maryanovich M, Sommerkamp
 P et al. Niche derived netrin-1 regulates hematopoietic stem cell dormancy via its
 receptor neogenin-1. Nat Commun 2021; 12: 608.
- 47 Boneschansker L, Nakayama H, Eisenga M, Wedel J, Klagsbrun M, Irimia D *et* 812 *al.* Netrin-1 Augments Chemokinesis in CD4+ T Cells In Vitro and Elicits a
- Proinflammatory Response In Vivo. *J Immunol* 2016; **197**: 1389–1398.
- Lu W, Yu W, He J, Liu W, Yang J, Lin X *et al.* Reprogramming
- 815 immunosuppressive myeloid cells facilitates immunotherapy for colorectal cancer.
- 816 *EMBO Mol Med* 2021; **13**. doi:10.15252/emmm.202012798.
- 49 Sung P-J, Rama N, Imbach J, Fiore S, Ducarouge B, Neves D *et al.* CancerAssociated Fibroblasts Produce Netrin-1 to Control Cancer Cell Plasticity. *Cancer*819 *Res* 2019; **79**: 3651–3661.
- Thakore PI, D'Ippolito AM, Song L, Safi A, Shivakumar NK, Kabadi AM *et al.*Highly specific epigenome editing by CRISPR-Cas9 repressors for silencing of distal
 regulatory elements. *Nat Methods* 2015; **12**: 1143–1149.
- Mieulet V, Garnier C, Kieffer Y, Guilbert T, Nemati F, Marangoni E *et al.*Stiffness increases with myofibroblast content and collagen density in mesenchymal
 high grade serous ovarian cancer. *Sci Rep* 2021; **11**: 4219.
- 52 Gibert B, Delloye-Bourgeois C, Gattolliat C-H, Meurette O, Le Guernevel S,
- Fombonne J *et al.* Regulation by miR181 family of the dependence receptor CDON
 tumor suppressive activity in neuroblastoma. *J Natl Cancer Inst* 2014; **106**.
- 829 doi:10.1093/jnci/dju318.

- Jiang S, Richaud M, Vieugué P, Rama N, Delcros J, Siouda M *et al.* Targeting netrin-3 in small cell lung cancer and neuroblastoma. *EMBO Mol Med* 2021. doi:10.15252/emmm.202012878.
- 833

836

837 Figure legends

838

Figure 1: Netrin-1 expression is a poor prognosis marker and correlates with stem cells marker in human breast and ovary cancers.

841 **A-B.** Kaplan-Meier curves representing the percentage of overall survival of breast 842 cancer patients (A, n=89) or treatment-free survival among patients with localized 843 disease (**B**, n=67). The cohort has been split in netrin-1 High (green) and Low (red) by 844 the median expression in their tumors. C. Netrin-1 mRNA expression level within 845 tumors segmented in High and Low by the median expression of CD44 mRNA (n=89). 846 Bars are mean values +/- s.e.m. **D.** Netrin-1 mRNA expression level measured in fresh 847 human tumors obtained at the diagnosis by g-RT-PCR. Each tumor was split to 848 analyze the bulk or their corresponding derived mammospheres (Spheres, n=12) after 849 dissociation. Bars are mean values +/- s.e.m. normalized with the tumor bulk group. 850 E-F. Kaplan-Meier curves representing the percentage of overall survival (E) or 851 treatment free survival (F) of high-grade ovarian carcinoma patients (n=51) with netrin-852 1 High (green), Low (red) Negative (black) expression in their tumors (Netrin-1 was 853 measured by immunohistochemistry (IHC)). Median netrin-1 expression was used as 854 a cutoff between groups. **G.** Boxplot presenting Allred score combining the percentage 855 of positive cells and signal intensity of netrin-1 measured by IHC in patient's responder 856 (n=17) vs resistant to platin treatment (n=8). H. Netrin-1 mRNA expression level in 857 tumors segmented on the CD44 mRNA expression from a cohort of High grade serous 858 ovarian carcinomas (n=31). High and Low have been determined respectively by the 859 upper and lower values separated by the median of mRNA expression measured by 860 q-RT-PCR. Bars are mean values +/- s.e.m. In all panels statistics are presented as * 861 *p*<0.05; ** *p*<0.01.

862

Figure 2: Netrin-1 expression is associated with CSCs in human cell lines and in a PDX model of breast cancer resistance to chemotherapy.

A. Netrin-1 mRNA expression level was measured by q-RT-PCR in HS578t (n=3) or SKBR7 (n=3) cell lines treated 48h with 1 μ M Adriamycin (Adria). Bars are mean values +/- s.e.m., normalized with the control group. **B**. Netrin-1 mRNA expression level was

measured by q-RT-PCR in BT474 (n=4 vs 5) or Hs578t (n=5) cell lines comparing 868 869 standard 2D culture and mammosphere (Sphere) 3D. Bars are mean values +/- s.e.m., 870 normalized with the control 2D group. C. Netrin-1 mRNA expression level was measured by q-RT-PCR in CD49f^{low} and CD49f^{high} Hs578t (n=4) sorted breast cell 871 population and in CD44^{low} and CD44^{high} OV90 ovary cell line (n=6 vs 3). Bars are mean 872 873 values +/- s.e.m., normalized with the control Low group. **D.** Tumor volume was 874 measured along the different phases of the HBC146 PDX breast cancer model³³ 875 treated with chemotherapy Adriamycin-Cyclophosphamide (AC): Growth, Regression, 876 Remission and Relapse (n=20). Samples were collected at the different phases (red 877 circles). E. Netrin-1 and CD44 mRNA expression levels were measured by g-RT-PCR 878 in the different phases (untreated n=8, regression n=8 and relapse n=10). Bars are 879 mean values +/- s.e.m., normalized with the untreated group. F. Percentage of CD44^{high} 880 cells counted by FACS analysis in untreated tumors compared to regressing tumors 881 (n=9 vs 3). G. Number of mammospheres formed by 1000 viable cells isolated from 882 untreated HBC146 tumors or at the regression phase (n=5 vs 9). Bars are mean values 883 +/- s.e.m. H. Netrin-1 mRNA expression level measured by g-RT-PCR in untreated 884 bulk tumors and mammospheres (n=7). Bars are mean values +/- SEM, normalized to 885 the bulk tumor group. I. SubG1 cell present in Hs578t cell line treated with siRNA 886 control (siCtr) or siRNA against Netrin-1(siNtn-1) (n=3). J. Cell viability measured by 887 WST1 assay in SKBR7 (n=3) cell lines treated with siCtr and siNetrin-1 alone or in 888 combination with Adriamycin. Bars are mean values +/- SEM, normalized to the siCtr 889 group (* are statistics against siCtr and + against siCtr + Adriamycin). K. Number of 890 mammospheres formed by 1000 viable cells of Hs578t (n=5) and OV90 (n=3) cell lines 891 treated with siCtr and siNetrin-1. Bars are mean values +/- SEM. In all panels statistics 892 are presented as * *p*<0.05; ** *p*<0.01; *** or *** *p*<0.001.

893

894 Figure 3: Netrin-1 blockade delays cancer relapse and impact CSCs.

A. Tumor volume was measured along the different phases of the HBC146 PDX model: Growth, Regression after chemotherapy (Adriamycin-Cyclophosphamide: AC), Remission and Relapse. At day 21, the mice were randomized into two groups which were intraperitoneally treated either by vehicle (n=20) or with 10mg/kg of anti-netrin1 antibody weekly (n=20). p=0.0002 by ANOVA on the tumor volume at the regression. B. Kaplan-Meier curves representing the percent of mice free of relapse in vehicle 901 (green) and anti-netrin-1 mAb (red) treated groups. The relapse volume threshold has 902 been determined at 40mm³. Median of relapse are respectively 56.0 and 80.5 days 903 after chemotherapy for the vehicle and anti-netrin-1 groups, p=0.0078 by Gehan-904 Breslow-Wilcoxon test. C. Number of mammospheres formed by 1000 viable cells (left) 905 or viable PI negative cells (right) isolated from HBC146 tumors treated with 906 chemotherapy alone or associated with anti-netrin-1 mAb. Samples were collected in 907 regressing tumors, below the volume threshold of 40mm³ (n=5 vs 9). Bars are mean 908 values +/- s.e.m. **D.** Representative CD44 immunohistochemistry analysis of HBC146 909 tumors treated with chemotherapy alone or associated with the anti-netrin-1 mAb. 910 Samples were collected in regressing tumors. Scale bar (represented by a line): 50µm. 911 E-F. Serial transplantation experiments of HBC146 (n=10 per group) and BRE-012 (n= 912 10 per group) breast PDX. First, tumors were treated with the anti-netrin-1 mAb or with 913 a control IgG1 and then regrafted in other mice. Secondary recipient were not treated 914 after the re-engraftment, the percentage of mice free of tumor is presented in both 915 groups. G. Tumor volume was measured in mice bearing the OV21 ovarian PDX model 916 and treated with Carboplatin/Paclitaxel (CP) chemotherapy and randomly separated in 917 two groups intraperitoneally treated either with control IgG1 (n=8) or with 10mg/kg of 918 anti-netrin1 antibody (n=8). (p=0.0101; ANOVA test). H-I. Serial transplantation 919 experiments of ovarian cancer cell lines OV90 (n=12) or OVCAR3 (n=8). First, grafted 920 tumors were treated with the anti-netrin-1 mAb or with a IgG1 control and after 921 dissociation, 1 x 10⁶ living cells were regrafted in new recipient mice Results are 922 presented as the percentage of mice free of tumor take with respectively. In all panels 923 statistics are presented as * *p*<0.05; ** *p*<0.01; *** *p*<0.001.

924

Figure 4: Netrin-1 blockade alleviates resistance to immune checkpoint inhibitoranti-CTLA-4.

927 A. Tumor growth quantification of syngeneic EMT6 mammary carcinoma, in mice 928 treated with control IgG1 or anti-netrin-1 mAb, in combination with anti-CTLA-4 mAb. 929 **B.** Kaplan-Meier survival analysis of the mice of **A**. after 80 days. **C**. Spider graph 930 presenting flow cytometry analysis of the immune infiltrate. All components of the 931 lymphoid panel were detected and quantified after EMT6 tumor dissociation and 932 isolation of cell population using Lymphocytes specific antibodies. D. Analysis of CD8+ 933 lymphocytes by flow cytometry infiltrating tumors treated with either anti-CTLA-4 mAb 934 combined or not with anti-netrin-1 mAb and compared to a IgG1 control (n=17 per

935 group). Results are the mean of 2 different experiments presented in **C**. and are 936 presented as a ratio on total CD45⁺ cells singlets for each tumor. **E**. Analysis of tumor 937 associated macrophages defined as CD11b⁺ LY6C⁻ F4/80⁺ cells (n=16 per group). 938 Results where the mean of 2 different experiments and are presented as a ratio on 939 total CD45⁺ cells singlets for each tumor. In all panels statistic are presented as * 940 p<0.05; ** p<0.01.

941

Figure 5: Netrin-1 blockade efficiently impacts CSCs/TICs untargeted by antiCTLA-4 mAb.

944 A-B. Flow cytometry analysis of PMN-MDSC obtained after dissociation of EMT6 (n=9 945 per group) or MMTV/NeuT (n=16 vs 19) tumors treated with either control isotype IgG1 946 or anti-netrin-1 mAb. PMN-MDSC are defined as CD11b⁺ Singlets⁺ cells among the CD45⁺ (CD11b⁺ Ly6G⁺ Ly6C^{interm}) population. **C**. EMT-6 tumor implanted in syngeneic 947 BALBc/J mice were treated with anti-netrin-1 and anti-CTLA4 and injected/not with 948 949 PMN-MDSC cells. **D**. Flow cytometry analysis of CD49f⁺ EMT6 cells in control parental 950 cells, or netrin-1 silenced by shRNA (Sh netrin-1) cocultured, or not, with PMN-MDSC 951 (n=3). E. Flow cytometry analysis of EMT6 cancer cells obtained after dissociation of 952 tumors treated with either three treatments of control IgG1, anti-netrin-1 mAb alone or 953 in combination with anti-CTLA-4 (respectively n=10, 8, 8 and 10). CSC markers are 954 defined as CD49f⁺ cells among the CD45^{neg} (CD45⁻) population. F. 955 Immunohistochemistry analysis and quantification of Ly6G⁺ cells in EMT6 obtained 956 after fixation of tumors treated with either three treatments of control IgG1, anti-netrin-957 1 mAb with or without anti-CTLA-4. n=8 field/tumor. G. Serial transplantation experiments with EMT6 cells in syngeneic BALBc/J mice. 5.10⁴ living cells, obtained 958 959 after the dissociation of tumors from mice treated with either control IgG1 vs. anti-960 netrin-1 mAb alone; or in combination with anti-CTLA4 (respectively n=7, 8, 8 and 8) 961 were grafted into recipient mice. Results are presented as percentage of mice free of 962 tumor. H. Serial transplantation of EMT6 cells were used as an indicator of tumor initiating potential of CD49f^{+/-} populations. Tumors were dissociated from EMT6 tumors 963 964 of mice treated with control IgG1 or anti-netrin-1 mAb. After dissociation, cells were 965 sorted based on CD49f expression and 5.10⁴ living cells of each positive or negative 966 populations were regrafted in new recipient animals (n=10 per group). Results are

- 967 presented as the percentage of mice free of tumor. In all panels statistics are presented
- 968 as * *p*<0.05; ** *p*<0.01; *** *p*<0.001.









- Cert 1







Supplementary Figure 2







Supplementary Figure 5