Cancer cell motility is a key driver of metastasis. Although the intravasation of cancer cells into the blood stream is highly dependent on their motility and metastatic dissemination is the primary cause of cancer related deaths, current therapeutic strategies do not target the genes and proteins that are essential for cell motility. A primary reason for this is because the identification of cell motility-related genes that are relevant in vivo requires the visualization of metastatic lesions forming in an appropriate in vivo model. The cancer research community has lacked an in vivo and intravital metastatic cancer model that could be imaged as motility developed, in real-time. To address this, we developed a novel quantitative in vivo screening platform based on intravitall imaging in shell-less ex ovo chick embryos. We applied this imaging approach to screen a human genome-wide short hairpin RNA library (shRNA) versus the highly motile head and neck cancer cells (HEp3 cell line) introduced into the chorionallantoic membrane (CAM) of chick embryos and identified multiple novel in vivo cancer cell motility-associated genes. When the expression of several of the identified genes was inhibited in the HEp3 tumors, we observed a nearly total block of spontaneous cancer metastasis.

INTRODUCTION
Currently, many cancer patients undergo chemotherapy, which is aimed at mitigating the risk of future metastasis. These treatments, however, have numerous system-wide off-target effects that negatively impact the patients’ quality of life. Although cancer cells are not recognized by the host immune system as “other” and so cannot be dependend on to potentiate an immunogenic response, cancer cells do differentially express genes compared to normal cells. We’ve found that this is the case in vivo with cancer cell motility-related genes. Given that the genes and signaling networks that drive in vivo motility and intravasation are different from those required for efficient primary tumor formation we sought to develop an in vivo approach to feasiably screen for genes required for motility, and therefore intravasation and metastasis. Metastasis is dependent on a tumor cell’s ability to intravasate, disseminate, evade immune detection, survive, proliferate, colonize elsewhere in the host, and do this repeatedly. We were able to visualize tumor colonies with expression-inhibited cell motility genes, and found that they form compact colonies instead of diffuse ones. This in vivo colony phenotype was distinctive enough to utilize this approach to screen for therapeutic targets of cell motility that would in turn impact intravasation and metastasis. Using our three-dimensional, intravital tumor-development screening approach in chick embryos to identify functional metastatic genes showed the importance of using an in vivo microenvironment because these phenotypes are not well recapitulated in two-dimensional culture.

INTRAVITAL IMAGING WITH EX OVO CHICK EMBRYOS AND IDENTIFYING METASTASIS GENES BY SCREENING A GENOME-WIDE RNA LIBRARY IN HEp3 CELLS
Structurally, the chicken embryo CAM is a highly vascularized, thin, transparent organ, surrounded by a dense collagen-rich matrix, very similar to mouse lung tissue. Human cancer cells robustly interact with the collagen fiber network in both tissues. These features make the CAM very advantageous for human cancer cell tumor development.

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because it can be easily visualized using \textit{in vivo} multiphoton imaging.

In our work, HEp3 cells were transformed with the 79 805-shRNA vector library so that each HEp3 cell contained a single shRNA vector. The transformed HEp3 cancer cells were then intravenously injected into the chick embryo where they disseminated throughout the vasculature. Roughly 10\% of the 25,000 injected tumor cells arrested individually in the CAM and extravasated into the extravascular stroma. They then proliferated into isolated, invasive metastatic colonies, each arising from a single transformed cell (Figure 1a).

Because thousands of individual metastatic colonies are visualized simultaneously in the CAM of a single embryo, we found that it was feasible to screen large libraries of genes by identifying phenotypic changes using intravitral microscopy. To screen the whole genome shRNA library containing 79,805 sequence-verified shRNAs targeting 30,728 human genes, we estimated that conducting the screen in 100 embryos would give us a minimum 3x coverage of the entire library.

METASTATIC AND BLOCKED METASTATIC COLONY PHENOTYPES
HEp3 cells are highly motile and as they proliferate in the CAM they form a “spread out” morphology because the cells migrate a significant distance from the point of extravasation. When their motility is reduced, however, the resulting colonies adopt a more compact morphology that was easily distinguished from those formed from highly motile HEp3 tumor cells. Since the \textit{ex ovo} chick embryo CAM is readily accessible, these compact tumour cell colonies were readily excised from the surrounding tissue for further analysis (Figure 1b, c). We utilized a high throughput sequencing approach to characterize the shRNAs present in each compact colony and identified eleven unique shRNA-targeted genes (ACTB, ACTC1, APBA2, C14orf142, KB-1460A1.5, KDELR3, KIAA0922, KIF3B, NR2F1, SRPK1, and TMEM229B) whose expression is associated with \textit{in vivo} cancer cell motility.

CANCER CELL-COLLAGEN MATRIX INTERACTION
When cancer cells invade into the stroma surrounding a tumor, they must first actively reorganize the collagen rich matrix at the tumor front to create areas of densely bundled, aligned collagen fibers. The resulting colonies adopt a more compact morphology that was easily distinguished from those formed from highly motile HEp3 tumor cells. Since the \textit{ex ovo} chick embryo CAM is readily accessible, these compact tumour cell colonies were readily excised from the surrounding tissue for further analysis (Figure 1b, c). We utilized a high throughput sequencing approach to characterize the shRNAs present in each compact colony and identified eleven unique shRNA-targeted genes (ACTB, ACTC1, APBA2, C14orf142, KB-1460A1.5, KDELR3, KIAA0922, KIF3B, NR2F1, SRPK1, and TMEM229B) whose expression is associated with \textit{in vivo} cancer cell motility.

CONCLUSIONS
Overall, we found an extraordinarily tight link between a gene’s \textit{in vivo} cancer cell motility phenotype and its requirement for successful metastatic spread. This demonstrates that our quantitative \textit{in vivo} imaging-based screen-
ing approach is a powerful tool to identify potential therapeutic targets for metastasis, paving the way for the development of new therapies to block this deadliest aspect of human cancer. By coupling the highly accessible ex ovo chick embryo tumor model with fluorescence time-lapse intravital imaging and high-throughput sequencing, we designed a rapid and quantitative method to detect clinically-relevant metastasis phenotypes. In particular, three of the genes discovered using this intravital imaging platform, KIF3B, SRPK1 and NR2F1, represented very promising therapeutic targets for metastasis. Future work would be to test these metastasis-associated genes and gene-products as drug targets aimed at blocking metastasis in vivo.

Therapies designed to specifically target the rate limiting steps of metastatic dissemination of tumor cells could significantly improve cancer treatment by directly blocking the systemic spread of cancer. Cancer treatment could also shift away from the use of chemotherapies with detrimental side-effects which would dramatically improve the quality of life of cancer patients.

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CONFLICT OF INTEREST
The authors declare no competing interests.
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